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Extremely sensitive, background-free gene detection using binary probes and Qβ replicase

(diagnostic clinical assays/isolation of probe-target hybrids/T4 DNA ligase/target-dependent signal generation/exponential amplification of reporter RNAs)

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ABSTRACT **We have developed a specific and sensitive nucleic acid amplification assay that is suitable for routine gene detection. The assay is based on a novel molecular genetic strategy in which two different RNA probes are hybridized to adjacent positions on a target nucleic acid and then ligated to form an amplifiable reporter RNA. The reporter RNA is then replicated up to a hundred billion-fold in a 30-min isothermal reaction that signals the presence of the target. The assay can detect fewer than 100 nucleic acid molecules; it provides quantitative results over a wide range of target concentrations and it employs a universal format that can detect any infectious agent.**

Extremely specific and sensitive assays are needed for the routine detection of rare pathogenic agents in clinical samples. The most specific assays employ single-stranded oligonucleotide probes to seek out and bind to unique regions of a pathogen's nucleic acid. The resulting probe–target hybrids are the most specific and stable intermolecular complexes known. When a high concentration of probes is incubated with a sample, virtually every target molecule forms a hybrid. One need only remove the probes that are not hybridized and then count the remaining hybridized probes to determine the degree of infection. However, the number of target molecules in a sample is often so low that classical detection schemes, such as labeling the probes with radioactive atoms or fluorescent moieties, are not sufficiently sensitive. Thus it is necessary to design assays in which a large number of reporter molecules are generated for every target that is present.

Although a very large number of copies are synthesized in target–sequence amplification assays (1–5), such as those that use the polymerase chain reaction, their design creates practical problems that restrict their use to specialized laboratories. These assays are usually carried out in crude cellular extracts, where they can be inhibited by cellular components and where the presence of unrelated nucleic acids can lead to false-positive signals; different sample preparation protocols are needed for different tissues and for different infectious agents; relatively expensive equipment is often required to alternately raise and lower the temperature; and additional steps are needed to detect the amplified nucleic acid, increasing the risk of contaminating other samples. Although there are a variety of solutions for each of these problems, the resulting assays are complex and difficult to quantitate.

We have been working on an alternative amplification scheme that avoids these problems. In our approach, the probes, rather than the targets, are amplified exponentially (6–8). Unlike target amplification schemes, where repeated cycles of hybridization and polymerization are carried out in the same solution, our assays employ a single hybridization step that is carried out under universal and highly stringent conditions. The probe–target hybrids are then isolated and the bound probes are amplified exponentially in a brief isothermal reaction. Our probes are recombinant RNAs (9), in which a probe sequence is embedded at an appropriate site within the sequence of MDV-1 RNA (10, 11), which is a naturally occurring template for the RNA replicase of bacteriophage Qβ. These recombinant RNAs hybridize to their targets as do ordinary probes, but unlike ordinary probes, more than a billion copies of each probe can be synthesized in a 30-min incubation with Qβ replicase (12). This amplification does not require primers, and strand separation occurs naturally at 37°C (13, 14). The large number of RNA molecules that are synthesized signals the presence of the target nucleic acid. Because a single probe molecule can initiate exponential amplification (15), these assays can be extremely sensitive. In practice, however, their sensitivity has been limited by how well the probe–target hybrids can be separated from the large number of nonhybridized probes that are present to ensure that hybridization occurs rapidly. Despite the use of reversible target capture (16), which is an extremely efficient hybrid isolation procedure, we found that 10,000 nonhybridized probes could not be removed, generating a background signal that obscured the presence of rare targets (8).

In this report, we describe a new strategy that solves the problem posed by the persistence of nonhybridized amplifiable probes. The probe molecules were redesigned so that they cannot be amplified unless they hybridize to their target. We divided the recombinant-RNA probes into two separate molecules, neither of which can be amplified by itself, because neither contains all the elements of sequence and structure that are required for replication. The division site is located in the middle of the embedded probe sequence. When these "binary probes" are hybridized to adjacent positions on their target, they can be joined to each other by incubation with an appropriate ligase, generating an amplifiable reporter RNA. Nonhybridized probes, on the other hand, because they are not aligned on a target, have a very low probability of being ligated. By combining this target-dependent ligation step with a new and simpler hybrid-isolation step, signal generation is strictly dependent on the presence of target molecules, no background signals are generated, and the resulting assays are extraordinarily sensitive.

MATERIALS AND METHODS

Synthetic Target Molecules. A cDNA encoding a portion of the polymerase gene of human immunodeficiency virus type 1 (HIV-1) strain NL4-3 (17) was subcloned between the *Hin*dIII and *Xma*I restriction sites in the polylinker of plasmid pGEM-4Z (Promega). The resulting plasmid was linearized by digestion with endonuclease *Sma*I and transcribed by incuba-

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tion with bacteriophage T7 RNA polymerase. The 875-nt transcripts contained nucleotides 4229–5091 of the HIV-1 (NL4-3) genome (listed in the GenBank data base as HIVNL43).

Binary Probes. Both the left and the right probe were prepared by transcription from DNA templates. The first 63 nt at the 5′ end of the left probe (shown in Fig. 1) were identical to the 5′-terminal sequence of MDV-1 (+) RNA; the next 6 nt served as a spacer (which is not essential); and the last 23 nt were complementary to nucleotides 4596–4618 of HIV-1 (NL4-3) RNA. The 3′ ends of the left probe transcripts naturally terminate in the hydroxyl group required for ligation. The first 19 nt at the 5′ end of the right probe (also shown in Fig. 1) were complementary to nucleotides 4577–4595 of HIV-1 (NL4-3) RNA; the next 10 nt served as a spacer (also nonessential); and the last 156 nt were identical to the 3′-terminal sequence of MDV-1 (+) RNA. Normally, the 5′ ends of the right probe transcripts would have contained a triphosphate group, which cannot participate in ligation; however, in addition to providing guanosine triphosphate as a precursor for transcription, we also provided a 20-fold excess of guanosine monophosphate, which is incorporated into the 5′-terminal position, assuring that almost all of the right probe transcripts contained the monophosphate group required for ligation. Many of the left probe transcripts possessed additional (nontemplated) nucleotides at their 3′ end. Electrophoretic isolation of correct-length left probe transcripts, though not done for the experiments reported here, improves ligation efficiency from 8% to 40%.

The DNAs used as templates for the synthesis of these probes were prepared in polymerase chain reactions initiated with plasmid, pT7MDV (18), which contains a cDNA copy of the MDV-1 sequence. The primers used in these reactions possessed sequences at their 5′ ends that added a T7 RNA polymerase promoter and a probe sequence to the amplified DNA. The first primer for the left probe template contained the promoter sequence and the other primer contained a portion of the target sequence, while the first primer for the right probe template contained the promoter sequence directed toward the complement of the remainder of the target sequence and the other primer was complementary to the 3′ end of the MDV-1 (+) sequence.

Capture Probes. These single-stranded oligodeoxynucleotides are complementary to the target (19) and possess a biotin moiety at their 5′ end that enables them to bind strongly to streptavidin (20), which is covalently linked to the surface of paramagnetic particles (21). The use of two different capture probes improves the efficiency of target capture by 60%. These oligonucleotides were prepared commercially (Operon Technologies, Alameda, CA) by chemical synthesis and contained 4 nt at their 5′ end that served as a spacer between the probe sequence and the 5′-terminal biotin moiety. The sequence of one capture probe was 5′-biotin-TACGATGTCTGTTGCTATTATGTCTACTATTCTTTCCCCTGCACTGTAC-3′, which is complementary to nucleotides 4808–4852 of HIV-1 (NL4-3) RNA; and the sequence of the other capture probe was 5′-biotin-TACGACTGCTACCAAGATAACTTTTCCTTCTAAATGTGTACAATCTAGC-3′, which is complementary to nucleotides 4415–4459 of HIV-1 (NL4-3) RNA.

HIV-1 Infected Lymphocytes. A suspension of H9 cells (22) was infected with HIV-1 isolate HTLV-III$_{MN}$ (23). Histological staining with serum from an HIV-positive individual, 24 hr after infection, indicated that more than 99% of the cells were infected. Stock samples were prepared by serially diluting the HIV-1-infected H9 cells with uninfected H9 cells. Each stock sample contained 600,000 cells. The cells were then washed and concentrated by centrifugation, dissolved in 240 μl of 5 M guanidine thiocyanate, and incubated at 37°C for 60 min. Concentrated solutions of this chaotropic salt lyse cells, inactivate enzymes, liberate nucleic acids from cellular matrices, unwind DNA molecules, and relax RNA secondary structures (24). Each lysate was mixed thoroughly and one sixth of its volume was assayed.

Assay Protocol. Some 10^{10} molecules of each binary probe and 10^{11} molecules of each capture probe were added to each sample. Hybrids were formed by incubation at 37°C for 60 min in 100 μl of buffer A [2 M guanidine thiocyanate (Fluka)/400 mM Tris·HCl, pH 7.5/5 mg of sodium N-lauroylsarcosine per ml/5 mg of bovine serum albumin fraction V per ml (Boehringer Mannheim)/and 80 mM EDTA] in polypropylene titer-

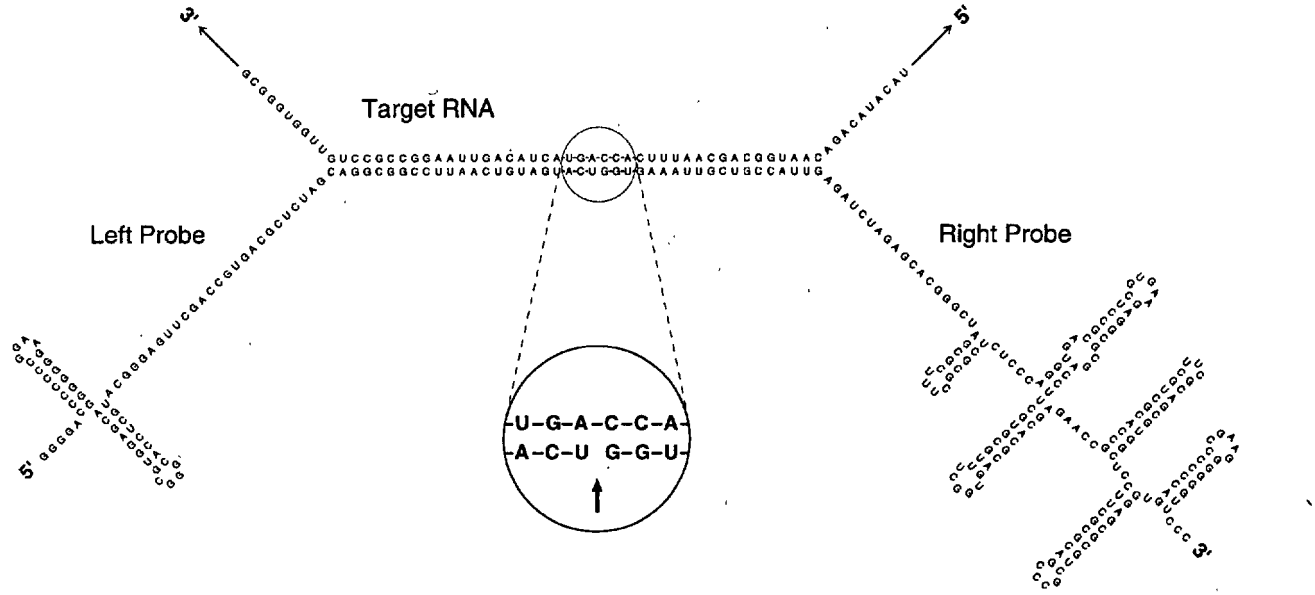

FIG. 1. Binary probes bound to a complementary HIV-1 target molecule. Neither the left probe nor the right probe can be amplified by incubation with Qβ replicase. However, if the binary probes are hybridized to adjacent positions on a target RNA (as shown), they can be ligated to each other to form a reporter RNA that can be amplified exponentially by Qβ replicase. The magnified view shows the phosphodiester bonds (short lines). An arrow points to the location where a phosphodiester bond will be formed when the hybrid is incubated with T4 DNA ligase. Ligated probes form a naturally occurring Qβ replicase template, MDV-1 RNA, containing an embedded HIV-1 probe sequence.

tubes (Bio–Rad). The presence of 2 M guanidine thiocyanate in the hybridization mixture promotes the formation of hybrids without interference from denatured cellular debris (25). The hybrids were then captured by adding 20 μl of a suspension of streptavidin-coated paramagnetic particles, as supplied by the manufacturer (Promega), and incubating at 37°C for 10 min. We discovered that the presence of 2 M guanidine thiocyanate does not prevent the biotinylated capture probes from binding to streptavidin. The particles were then washed four times with 200 μl of buffer A at 37°C to remove excess probes and cellular material, and washed an additional four times at 37°C with 200 μl of buffer B [5 mM $MgCl_2$/66 mM Tris·HCl, pH 7.5/1 mM ATP/0.5 mg of Nonidet P-40 per ml (Sigma)/1 mM dithiothreitol] to remove the guanidine thiocyanate. During each wash cycle, the suspended particles were agitated vigorously on a multitube vortex-type mixer (American Hospital Supply, McGaw Park, IL); they were then drawn to the walls of the titertube with the aid of a magnetic separation device (Vysis, Downers Grove, IL); and the wash solution was withdrawn by aspiration and replaced with a new solution. After the last wash, the particles were suspended in 50 μl of buffer B containing 1 unit of *Escherichia coli* ribonuclease H (Pharmacia) and incubated at 37°C for 10 min to release the hybrids. The particles were then magnetically drawn to the sides of the titertube and the supernatant containing the hybrids (≈45 μl) was transferred to a new titertube. Ligation was carried out by adding 5 μl of buffer B containing 25 units of bacteriophage T4 DNA ligase (Boehringer Mannheim) and incubating at 37°C for 60 min. The resulting reporter RNAs were exponentially amplified by adding 100 μl of buffer C (15 mM $MgCl_2$/45 mM Tris·HCl, pH 8/100 μM ATP/600 μM [α-^{32}P]CTP/600 μM GTP/and 600 μM UTP) containing 6 μg of Qβ replicase (Vysis) and incubating at 37°C for 31 min. Samples (5 μl) of each amplification reaction were withdrawn every minute (beginning at 8 min) and were added to 100 μl of a stop solution containing 20 mM EDTA (pH 8) and 120 mM NaCl. The RNA in each sample was precipitated by adding 400 μl of a solution containing 360 mM phosphoric acid, 20 mM sodium pyrophosphate, and 2 mM EDTA. The precipitated RNA was bound to a Zeta–Probe nylon membrane (Bio–Rad) on a dot-blot vacuum filtration manifold (Bio–Rad). The membrane was then washed with 500 ml of the precipitation solution to remove unincorporated [^{32}P]CTP. Finally, the membrane was air-dried and the [^{32}P]RNA present in each sample was visualized by autoradiography.

RESULTS

Design of the Assay. Our previous probe-amplification assays used recombinant RNAs that consisted of a probe sequence embedded within a template for Qβ replicase (8). To obtain hybridization probes that cannot be exponentially amplified, we divided the recombinant RNA probes into two separate molecules. The division site was located approximately in the middle of the embedded probe sequence (Fig. 1). To be replicated exponentially, an RNA must possess an internal replicase binding site (26), a particular 3′-terminal sequence for the initiation of replication (27), and a particular 5′-terminal sequence that encodes the 3′-terminal initiation sequence needed for the replication of the complementary strand (28). Neither fragment of the recombinant RNA probe possessed all of these sequences, and preliminary experiments confirmed that neither fragment could be replicated exponentially. However, each fragment retained the ability to hybridize to the target. When these binary probes are hybridized to a target strand, the partial probe sequence at the 3′ end of one molecule is brought immediately adjacent to the partial probe sequence at the 5′ end of the other molecule. Incubation of the hybrid with a target-dependent ligase covalently links the two probes, generating an amplifiable reporter RNA. Although other researchers obtained contrary results (29–31), we found that RNA fragments hybridized to an RNA target can be ligated efficiently by incubation with T4 DNA ligase. Consequently, we were able to design an assay in which nonamplifiable RNA fragments are used as probes and the generation of exponentially amplifiable reporter RNAs is strictly dependent on the presence of target strands.

The assay is shown schematically in Fig. 2. The sample is first dissolved in a guanidine thiocyanate solution. Binary probes and biotinylated DNA capture probes are added, and the

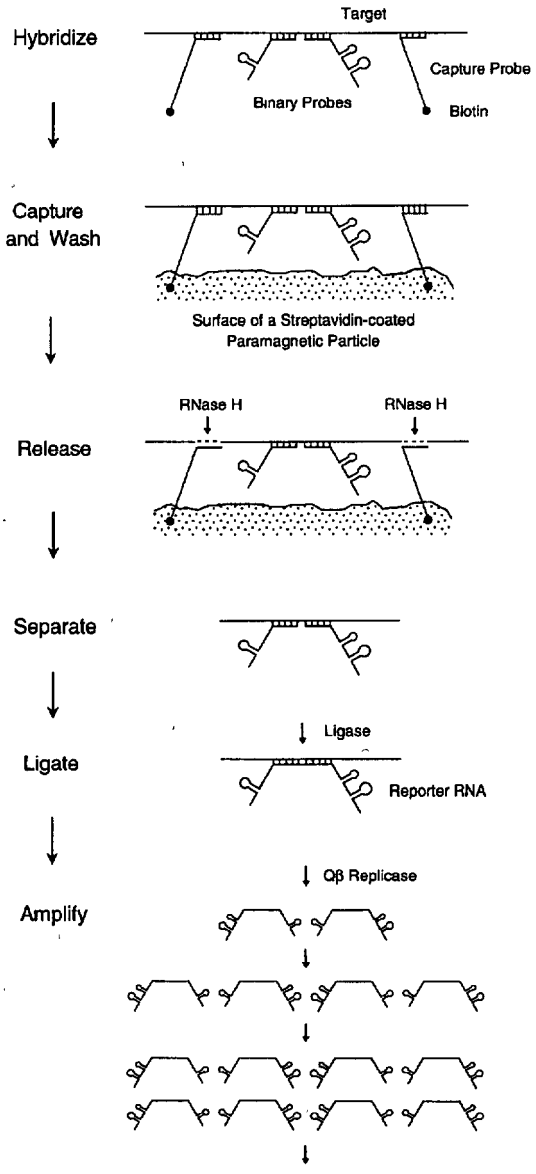

FIG. 2. Schematic representation of the key physical and enzymatic steps used in the assay. Both the target and the binary probes are RNA molecules. The capture probes are DNA molecules. Two different capture probes are used to increase the efficiency of capture. They hybridize to the target RNA on opposite sides of the sequence to which the binary probes are bound. Incubation with ribonuclease H digests the target RNA where it is bound to each capture probe, selectively releasing the binary probe–target hybrid from the surface of the paramagnetic particle. After removal of the particle with a magnet, the isolated hybrid is incubated with T4 DNA ligase (which serves here as an RNA-dependent RNA ligase), resulting in the formation of a reporter RNA that is then amplified exponentially by incubation with Qβ replicase.

mixture is incubated to form hybrids, which are then collected on the surface of streptavidin-coated paramagnetic particles. The particles are washed to remove excess probes and cellular material. Despite vigorous washing, some probes remain bound in a nonspecific manner to the walls of the assay tube, to the surface of the particles, and to the capture probes (32). Since nonhybridized binary probes can be ligated through chance alignment, it is necessary to further reduce their concentration. Therefore, the hybrids are separated from the nonspecifically bound probes by incubating the particles with ribonuclease H, which cleaves the target RNA where it is bound to the DNA capture probes, selectively releasing the probe–target hybrids into solution. The particles are then magnetically drawn aside and the supernatant containing the hybrids is transferred to a new tube. The isolated hybrids are then incubated with T4 DNA ligase to covalently link the binary probes, forming amplifiable reporter RNAs. When Qβ replicase is added, the only RNA that is synthesized is derived from binary probes that were hybridized to target strands.

In this assay format, hybridization precedes amplification. This is fundamentally different from the format used in target-sequence amplification assays, where repeated cycles of hybridization and amplification are carried out in the same solution. Because the nonenzymatic steps in our assay (sample preparation, hybridization, capture, and washing) are carried out in the presence of guanidine thiocyanate, which is an extremely effective denaturant, the same protocol can be used for all samples, irrespective of the type of tissue being tested or the nature of the suspected infectious agent. Furthermore, the enzymatic steps (hybrid release, ligation, and amplification) are deferred until the hybrids have been isolated and placed in a defined environment; thus they cannot be inhibited by cellular components, and false-positive signals cannot arise from the presence of irrelevant nucleic acids.

Assay with Simulated HIV-1 mRNA Targets. To determine the sensitivity of the assay, eight samples were prepared, each containing a different number of transcripts of the HIV-1 integrase gene. We used binary probes (shown in Fig. 1) that were complementary to a conserved sequence within the integrase gene and capture probes that were complementary to sequences on either side of the target sequence. Probe–target hybrids were formed, bound to the surface of paramagnetic particles, washed vigorously, and released into solution by digestion with ribonuclease H. The isolated hybrids were incubated with T4 DNA ligase and then incubated with radioactive nucleotides and Qβ replicase. Aliquots of each amplification reaction were taken at 1-min intervals, and the RNA in each aliquot was bound to a nylon membrane and visualized by autoradiography. The results are shown in Fig. 3. Reporter RNA was synthesized in the amplification reactions from samples that contained 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 HIV-1 target molecules. However, no reporter RNA was synthesized in the amplification reaction from the sample that contained 10 target molecules, nor was any RNA synthesized in the amplification reaction from the sample that contained no target molecules, even after 31 min of incubation. These results demonstrate that the sensitivity of the assay lies between 10 and 100 target molecules.

Because there is no background reaction, the sensitivity of the assay is not limited by the occurrence of obscuring signals. Instead, it is determined by the efficiency of the individual steps required to generate a reporter RNA. In experiments that followed the fate of labeled target strands and labeled probes through the various steps of the assay, we measured the number of hybrids that survived each step and found the following efficiencies: hybridization and capture, 95%; washing, 56%; hybrid release, 60%; and ligation, 8%—resulting in an overall efficiency of 2.6%. Thus, for every 100 target molecules in a sample, two or three reporter RNAs were formed, and they were exponentially amplified by Qβ replicase to generate a detectable signal. Samples containing less than 40 target molecules were unlikely to generate even a single molecule of amplifiable RNA.

Assay with HIV-1 Infected Human Lymphocytes. To demonstrate the specificity of the assay and to confirm that the presence of cellular material in the sample does not compromise sensitivity, seven samples were prepared by mixing HIV-1-infected lymphocytes with uninfected lymphocytes. Although each sample contained a different number of infected cells, the total number of cells in each was 100,000. A mock sample was also prepared by adding 10^6 HIV-1 transcripts to a lysate from 100,000 uninfected cells. The same protocol and probes were used as in the assays with HIV-1 transcripts. The results are shown in Fig. 4. Every sample that contained infected cells gave a clear signal, including the sample that contained only a single infected cell in the presence of 100,000 uninfected cells; yet the sample that did not contain any infected cells gave no signal, despite the presence of cellular components and nucleic acids from 100,000 uninfected cells. These results demonstrate that the assay is highly specific for the presence of target nucleic acid.

The results also illustrate how kinetic data can be used to determine the number of target molecules in an unknown sample. Because the amount of reporter RNA doubles at regular intervals, it takes longer for a given (arbitrary) amount of RNA to be synthesized in a reaction initiated with less RNA. We measured the amount of time it took for 100 ng of reporter RNA (6.4×10^{11} molecules) to be synthesized in each of the reactions shown in Fig. 4. This amount of RNA was just enough to be visible in the autoradiogram. We then plotted these "response times" against the number of infected cells in each sample. The results (Fig. 5) demonstrate that response time is inversely proportional to the logarithm of the number of targets. For every 10-fold decrease in the number of infected

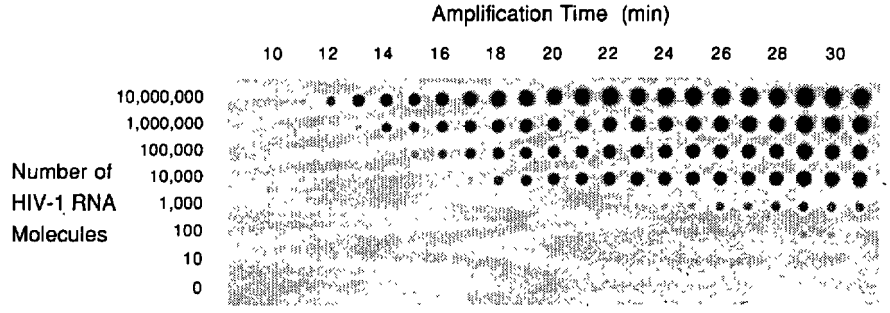

FIG. 3. Demonstration that binary probe assays are background-free and sufficiently sensitive to detect 100 target molecules. Each amplification reaction was sampled at 1-min intervals. The fewer the number of target molecules in the original sample, the longer it took before sufficient reporter RNA was synthesized for it to be visible in the autoradiogram. Measurements of the amount of radioactive reporter RNA synthesized in the reaction from the sample containing 100 molecules indicated that it was amplified a hundred billion-fold.

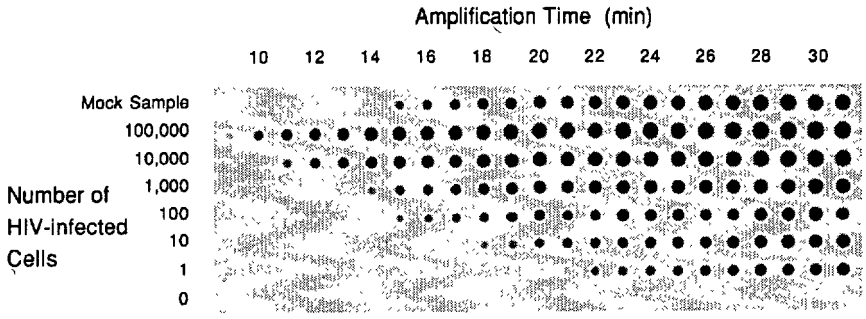

FIG. 4. Demonstration that binary probe assays are highly specific. Every sample that contained HIV-1-infected lymphocytes gave a positive signal, including the sample that contained only one infected cell in 100,000 uninfected cells; yet the sample that contained only uninfected cells gave no signal at all.

cells, it took about 2.1 min longer to synthesize 100 ng of reporter RNA. This relationship holds over an extremely wide range of target concentrations, extending from 1 cell to at least 100,000 cells. Thus, the number of infected cells in an unknown sample can be determined by comparing its response time to the results obtained from a set of reference standards.

DISCUSSION

Both of the enzymatic steps that occur before amplification are necessary. When the ligaton step is omitted, the number of reporter RNAs generated is five orders of magnitude lower. The only reason that any reporter RNAs occur in the absence of ligation is that Qβ replicase can occasionally continue polymerization across the gap in the ligation junction. When the enzymatic hybrid isolation step is omitted, the assays are no longer background-free because persistent nonhybridized binary probes on the surface of the particles are occasionally ligated to each other. However, target-independent ligation is second order and depends on the concentration of the probes. When the hybrids are isolated, the concentration of these probes is reduced to such a low level that not even a single reporter RNA is generated.

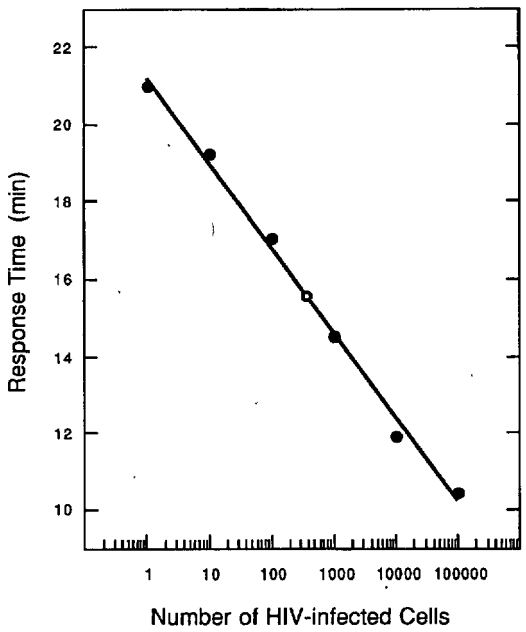

FIG. 5. Linear relationship between response time and the logarithm of the number of targets in a sample. The response time was measured for each amplification reaction shown in Fig. 4 and plotted against the number of HIV-1-infected lymphocytes in the corresponding sample. For every 10-fold decrease in the number of infected cells, it took about 2.1 min longer to synthesize 100 ng of reporter RNA. The response time of the mock sample (indicated by an open circle) corresponded to the response time that would have been obtained from a sample containing 360 infected cells. Because the mock sample contained 10^6 HIV-1 transcripts, we infer that each infected cell contained ≈2800 HIV-1 target molecules.

Several modifications will expand the utility of the assay. Virtually the only nucleic acid that is present in the amplification reactions is the reporter RNA. Therefore, the kinetic course of the reactions can be followed in real-time by including an intercalating fluorescent dye, such as propidium iodide, in the reaction mixture and measuring the increase in its fluorescence as it binds to the RNA being synthesized (33). Because there is no need to isolate the amplified RNA to detect it, the reaction tubes can be permanently sealed, eliminating the risk of contaminating other samples.

Binary probes that are specific for different target RNAs can be combined in a single assay tube, thus enabling the simultaneous detection of entire panels of pathogens. When these assays give a positive signal, the responsible pathogen can be identified because the amplified reporter RNA contains a unique embedded probe sequence. We recently devised novel nucleic acid detector probes, called "molecular beacons," that only fluoresce when they hybridize to their target (34). A series of molecular beacons, each specific for a different embedded probe sequence and each labeled with a fluorophore of a different color, can be included in an amplification reaction, enabling homogeneous, real-time detection in a multiplex format.

Binary probe assays are particularly amenable to distinguishing genetic alleles. Efficient ligation only occurs when the terminal nucleotides on either side of the ligation junction are correctly base-paired to the target strand (35). The occurrence of a terminal mismatch (due to an allelic difference) will result in a marked reduction in the number of amplifiable reporter RNAs. In these assays, the targets will be DNA, the ligation reaction will utilize an RNA–DNA heteroduplex (36), and selective hybrid release will be achieved by incubation with an appropriate restriction endonuclease.

Finally, binary probe assays will require little in the way of instrumentation. They can be carried out in a hermetically sealed device containing two reaction chambers, where hybridization, capture, washing, and hybrid release occur in one chamber, and ligation, amplification and signal detection occur in the other. Because these assays are simple in design and practice, they can routinely be used for gene detection.

We dedicate this paper to the memory of Sol Spiegelman, who first conceived quantitative nucleic acid hybridization assays (37, 38) and who introduced an entire generation of molecular biologists to the exponential amplification of nucleic acids (39). We thank Claire Grigaux for her expert technical assistance; David Ho, William

Honnen, and Abraham Pinter for the preparation of HIV-1-infected lymphocytes; and Harvey Bialy, Herman Blok, Karl Drlica, and David Yong Zhang for their incisive observations. This research was supported by the National Institutes of Health (Grants HL-43521 and AI-37015) and the American Foundation for AIDS Research (Grant 02063-15-RGR). U.L. is supported by the Beijer Foundation, and P.M.L. is supported by an International Research Scholars Award from the Howard Hughes Medical Institute.

1. Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. & Arnheim, N. (1985) *Science* **230,** 1350–1354.
2. Mullis, K. B. & Faloona, F. A. (1987) *Methods Enzymol.* **155,** 335–350.
3. Guatelli, J. C., Whitfield, K. M., Kwoh, D. Y., Barringer, K. J., Richman, D. D. & Gingeras, T. R. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1874–1878.
4. Barany, F. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 189–193.
5. Walker, G. T., Fraiser, M. S., Schramm, J. L., Little, M. C., Nadeau, J. G. & Malinowski, D. P. (1992) *Nucleic Acids Res.* **20,** 1691–1696.
6. Chu, B. C. F., Kramer, F. R. & Orgel, L. E. (1986) *Nucleic Acids Res.* **14,** 5591–5603.
7. Kramer, F. R. & Lizardi, P. M. (1989) *Nature (London)* **339,** 401–402.
8. Lomeli, H., Tyagi, S., Pritchard, C. G., Lizardi, P. M. & Kramer, F. R. (1989) *Clin. Chem.* **35,** 1826–1831.
9. Miele, E. A., Mills, D. R. & Kramer, F. R. (1983) *J. Mol. Biol.* **171,** 281–295.
10. Kacian, D. L., Mills, D. R., Kramer, F. R. & Spiegelman, S. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 3038–3042.
11. Mills, D. R., Kramer, F. R. & Spiegelman, S. (1973) *Science* **180,** 916–927.
12. Lizardi, P. M., Guerra, C. E., Lomeli, H., Tussie-Luna, I. & Kramer, F. R. (1988) *Bio/Technology* **6,** 1197–1202.
13. Dobkin, C., Mills, D. R., Kramer, F. R. & Spiegelman, S. (1979) *Biochemistry* **18,** 2038–2044.
14. Kramer, F. R. & Mills, D. R. (1981) *Nucleic Acids Res.* **19,** 5109–5124.
15. Levisohn, R. & Spiegelman, S. (1968) *Proc. Natl. Acad. Sci. USA* **60,** 866–872.
16. Morrissey, D. V., Lombardo, M., Eldredge, J. K., Kearney, K. R., Groody, E. P. & Collins, M. L. (1989) *Anal. Biochem.* **81,** 345–359.
17. Adachi, A., Gendelman, H. E., Koenig, S., Folks, T., Willey, R., Rabson, A. & Martin, M. A. (1986) *J. Virol.* **59,** 284–291.
18. Axelrod, V. D., Brown, E., Priano, C. & Mills, D. R. (1991) *Virology* **184,** 595–608.
19. Syvänen, A.-C., Laaksonen, M. & Söderlund, H. (1986) *Nucleic Acids Res.* **14,** 5037–5048.
20. Langer, P. R., Waldrop, A. A. & Ward, D. C. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 6633–6637.
21. Uhlen, M. (1989) *Nature (London)* **340,** 733–734.
22. Popovic, M., Read-Connole, E. & Gallo, R. C. (1984) *Lancet* **ii,** 1472–1473.
23. Shaw, G. M., Hahn, B. H., Arya, S. K., Groopman, J. E., Gallo, R. C. & Wong-Staal, F. (1984) *Science* **226,** 1165–1171.
24. Pelligrino, M. G., Lewin, M., Meyer, W. A., III, Lanciotti, R. S., Bhaduri-Hauck, L., Volsky, D. J., Sakai, K., Folks, T. M. & Gillespie, D. (1987) *BioTechniques* **5,** 452–460.
25. Thompson, J. & Gillespie, D. (1987) *Anal. Biochem.* **163,** 281–291.
26. Nishihara, T., Mills, D. R. & Kramer, F. R. (1983) *J. Biochem. (Tokyo)* **93,** 669–674.
27. Mills, D. R., Kramer, F. R., Dobkin, C., Nishihara, T. & Cole, P. E. (1980) *Biochemistry* **19,** 228–236.
28. Weissmann, C., Feix, G. & Slor, H. (1968) *Cold Spring Harbor Symp. Quant. Biol.* **33,** 83–100.
29. Kleppe, K., van de Sande, J. H. & Khorana, H. G. (1970) *Proc. Natl. Acad. Sci. USA* **67,** 68–73.
30. Fareed, G. C., Wilt, E. M. & Richardson, C. C. (1971) *J. Biol. Chem.* **246,** 925–932.
31. Sano, H. & Feix, G. (1974) *Biochemistry* **13,** 5110–5115.
32. Shah, J. S., Liu, J., Smith, J., Popoff, S., Radcliffe, G., O'Brien, W. J., Serpe, G., Olive, D. M. & King, W. (1994) *J. Clin. Microbiol.* **32,** 2718–2724.
33. Burg, J. L., Cahill, P. B., Kutter, M., Stefano, J. E. & Mahan, D. E. (1995) *Anal. Biochem.* **230,** 263–272.
34. Tyagi, S. & Kramer, F. R. (1996) *Nat. Biotechnol.* **14,** 303–308.
35. Landegren, U., Kaiser, R., Sanders, J. & Hood, L. (1988) *Science* **241,** 1077–1080.
36. Moore, M. J. & Sharp, P. A. (1992) *Science* **256,** 992–997.
37. Hall, B. D. & Spiegelman, S. (1961) *Proc. Natl. Acad. Sci. USA* **47,** 137–146.
38. Gillespie, D. & Spiegelman, S. (1965) *J. Mol. Biol.* **12,** 829–842.
39. Haruna, I. & Spiegelman, S. (1965) *Science* **150,** 884–886.

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Review

Molecular diagnostics of infectious diseases

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Over the past several years, the development and application of molecular diagnostic techniques has initiated a revolution in the diagnosis and monitoring of infectious diseases. Microbial phenotypic characteristics, such as protein, bacteriophage, and chromatographic profiles, as well as biotyping and susceptibility testing, are used in most routine laboratories for identification and differentiation. Nucleic acid techniques, such as plasmid profiling, various methods for generating restriction fragment length polymorphisms, and the polymerase chain reaction (PCR), are making increasing inroads into clinical laboratories. PCR-based systems to detect the etiologic agents of disease directly from clinical samples, without the need for culture, have been useful in rapid detection of unculturable or fastidious microorganisms. Additionally, sequence analysis of amplified microbial DNA allows for identification and better characterization of the pathogen. Subspecies variation, identified by various techniques, has been shown to be important in the prognosis of certain diseases. Other important advances include the determination of viral load and the direct detection of genes or gene mutations responsible for drug resistance. Increased use of automation and user-friendly software makes these technologies more widely available. In all, the detection of infectious agents at the nucleic acid level represents a true synthesis of clinical chemistry and clinical microbiology techniques.

Over the past century microbiologists have searched for more rapid and efficient means of microbial identification. The identification and differentiation of microorganisms has principally relied on microbial morphology and growth variables. Advances in molecular biology over the past 10 years have opened new avenues for microbial identification and characterization [1–5].

The traditional methods of microbial identification rely solely on the phenotypic characteristics of the organism. Bacterial fermentation, fungal conidiogenesis, parasitic morphology, and viral cytopathic effects are a few phenotypic characteristics commonly used. Some phenotypic characteristics are sensitive enough for strain characterization; these include isoenzyme profiles, antibiotic susceptibility profiles, and chromatographic analysis of cellular fatty acids [6–13]. However, most phenotypic variables commonly observed in the microbiology laboratory are not sensitive enough for strain differentiation. When methods for microbial genome analysis became available, a new frontier in microbial identification and characterization was opened.

Early DNA hybridization studies were used to demonstrate relatedness amongst bacteria. This understanding of nucleic acid hybridization chemistry made possible nucleic acid probe technology [14–25]. Advances in plasmid and bacteriophage recovery and analysis have made possible plasmid profiling and bacteriophage typing, respectively [26–31]. Both have proven to be powerful tools for the epidemiologist investigating the source and mode of transmission of infectious diseases [26, 28, 30, 32–40]. These technologies, however, like the determinations of phenotypic variables, are limited by microbial recovery and growth.

Nucleic acid amplification technology has opened new avenues of microbial detection and characterization [1, 5, 41], such that growth is no longer required for microbial identification [42–52]. In this respect, molecular methods have surpassed traditional methods of detection for many fastidious organisms. The polymerase chain reaction (PCR) and other recently developed amplification techniques have simplified and accelerated the in vitro process of nucleic acid amplification. The amplified products, known as amplicons, may be characterized by various methods, including nucleic acid probe hybridization, analysis of fragments after restriction endonuclease digestion, or direct sequence analysis. Rapid techniques of nucleic acid amplification and characterization have significantly broadened the microbiologists' diagnostic arsenal.

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Traditional Microbial Typing

BIOTYPING

Traditional microbial identification methods typically rely on phenotypes, such as morphologic features, growth variables, and biochemical utilization of organic substrates. The biological profile of an organism is termed a biogram. The determination of relatedness of different organisms on the basis of their biograms is termed biotyping. Investigators must determine which profile variables have the greatest differentiating capabilities for a given organism [53, 54]. For example, gram stain characteristics, indole positivity, and the ability to grow on MacConkey medium do not aid in the differentiation of nonenterohemorrhagic *Escherichia coli* from *E. coli* O157:H7. However, sorbitol fermentation has proven to be an extremely useful characteristic of the biochemical profile used to differentiate these strains.

Biograms that are identical have been used to infer relatedness between strains in epidemiological investigations [32, 55, 56]. The biograms of organisms are not entirely stable, and several isotypes may exist from a single isolate [12]. Biograms may be influenced by genetic regulation, technical manipulation, and the gain or loss of plasmids. In many instances, biotyping is used in conjunction with other methods to more accurately profile microorganisms [32].

ANTIBIOGRAMS, RESISTOGRAMS, AND BACTERIOCIN TYPING

The susceptibility or resistance of an organism to a possibly toxic agent forms the basis of the following typing techniques. The antibiogram is the susceptibility profile of an organism to a variety of antimicrobial agents, whereas the resistogram is the susceptibility profile to dyes and heavy metals [26]. Bacteriocin typing is the susceptibility of the isolate to various bacteriocins, i.e., toxins that are produced by a collected set of producer strains These three techniques are limited by the number of agents tested per organism.

By far, the antibiogram is the most commonly used susceptibility/resistance typing technique, most probably because the data required for antibiogram analysis are available routinely from the antimicrobial susceptibility testing laboratory. Although antibiograms have been used successfully to demonstrate relatedness, this technology is limited [6, 10, 55]. And although organisms with similar antibiograms may be related, such is not necessarily the case. The antibiogram of an organism is not always constant [57]. Selective pressure from antimicrobial therapy may alter an organism's antimicrobial susceptibility profile [58], such that related organisms show different resistance profiles. These alterations may result from chromosomal point mutations or from the gain or loss of extrachromosomal DNA such as plasmids or transposons [26, 57, 59].

PROTEIN ANALYSIS

Commercially available antibodies are routinely used to specifically identify antigenic proteins from a wide variety of organisms. In some instances, the test may be used only to identify the genus and species of an organism. Examples of this include the cryptococcal antigen agglutination assay and the exoantigen assay for *Histoplasma capsulatum*. Other immunoassays are designed to subtype microbes [60]. Monoclonal antibodies directed against the major subtypes of the influenza virus, as well as the various serotypes of *Salmonella*, are commonly used in speciation. Specific antigenic proteins may be detected by antibodies directed against these proteins in immunoblot methods [12, 61].

Electrophoretic typing techniques have been used to examine outer membrane proteins, whole-cell lysates, and particular enzymes [6, 55]. Several electrophoretic methods are available to examine the protein profile of an organism. Generally, outer membrane proteins and proteins from cell lysates are examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. This technique denatures the proteins and separates them on the basis of molecular mass. The protein profile may be used to compare strains [8, 55, 62].

Nondenaturing conditions are used for the electrophoretic separation of active enzymes. Multilocus enzyme electrophoresis is the typing technique based on the electrophoretic pattern of several constitutive enzymes [63]. Differences in electrophoretic migration of functionally similar enzymes (e.g., lactate dehydrogenase isoenzymes) represent different alleles. These differences or similarities, especially when numerous enzymes are examined, may be used to exclude or infer relatedness [6, 8, 10].

The results of these studies may be difficult to interpret, however. The absence of a particular protein may simply reflect downregulation of that particular gene product, rather than the loss of that particular gene. Additionally, the electrophoretic migration of proteins is dependent on molecular mass, net protein charge, or both. Mutations that do not alter these characteristics will not be detected.

PHAGE ANALYSIS

Bacteriophages, viruses that infect and lyse bacteria, are often specific for strains within a species. A collection of bacteriophages, many of which often infect similar bacteria, is termed a panel. When a bacterial isolate is exposed to a panel of bacteriophages, a profile is generated—a listing of which bacteriophages are capable of infecting and lysing the bacteria. The bacteriophage profile may be used to type bacterial strains within a given species [31, 62]. The more closely related the bacterial strains, the greater the similarity of the bacteriophage profiles. Bacteriophage profiles have been used successfully to type various organisms associated with epidemic outbreaks [64, 65]. However, this typing method is labor-intensive

and requires the maintenance of bacteriophage panels for a wide variety of bacteria. Additionally, bacteriophage profiles may fail to identify isolates, are often difficult to interpret, and may give poor reproducibility [62].

CHROMATOGRAPHIC ANALYSIS

Chromatographic analysis of short-chain fatty acid production is a routine method used to aid in the identification of anaerobic bacteria. Computer-aided gas–liquid chromatography is commercially available and is a means of microbial identification. This identification system utilizes the type and amount of cellular fatty acids present in the lysate of an organism. Many species have unique cellular fatty acid chromatographic profiles [9, 13]. Relationships between strains of a particular species may be inferred from highly similar cellular fatty acid profiles [7].

Chromatographic analysis is reliable when organisms are grown under identical conditions and the cellular fatty acids are extracted without technical variation. These constraints, however, limit the accuracy of this technology with respect to strain and in some instances even species-level identification.

Nucleic Acid-Based Typing Systems

PLASMID ANALYSIS

Plasmids are small, self-replicating circular DNA found in many bacteria. These often encode genes related to antibiotic resistance and certain virulence factors. In epidemiological studies, relatedness of isolated pathogenic bacterial strains can be determined from the number and size of plasmids the bacteria carry. Plasmid profile analysis was among the earliest nucleic acid-based techniques applied to the diagnosis of infectious diseases and has proven useful in numerous investigations [26–30, 60]. This method has also been widely utilized for tracking antimicrobial resistance during nosocomial outbreaks [26, 66, 67]. In studies of the epidemiology of plasmids, analysis of restriction fragments has proved valuable. This technique is widely used to monitor the spread of resistance-encoding plasmids between organisms and between hospitals, communities, or even countries [37–40]. The weakness of the analysis is inherent in the fact that plasmids are mobile, extrachromosomal elements, not part of the chromosomal genotype. Because plasmids can be spontaneously lost from or readily acquired by a host stain, epidemiologically related isolates can exhibit different plasmid profiles [68].

RESTRICTION ENZYME PATTERN

Restriction endonucleases recognize specific nucleotide sequences in DNA and produce double-stranded cleavages that break the DNA into small fragments. The number and sizes of the restriction fragments, called restriction fragment length polymorphisms (RFLPs)[1], generated by digesting microbial DNA are influenced by both the recognition sequence of the enzyme and the composition of the DNA. In conventional restriction endonuclease analysis, chromosomal or plasmid DNA is extracted from microbial specimens and then digested with endonucleases into small fragments. These fragments are then separated by size with use of agarose gel electrophoresis. The nucleic acid electrophoretic pattern can then be visualized by ethidium bromide staining and examination under UV light.

Restriction endonuclease analysis has the advantage of being highly reproducible, very accurate in determining the relatedness of microbial strains, and well within the technical capabilities of experienced laboratory technologists. However, the major limitation of this technique, especially for chromosomal DNA, is the difficulty of comparing the complex profiles generated, which consist of hundreds of fragments. To address this problem, pulse-field gel electrophoresis (PFGE) has been developed [69] to enable the separation of large DNA fragments. PFGE provides a chromosomal restriction profile typically composed of 5 to 20 distinct, well-resolved fragments ranging from ~10–800 kilobases (kb) [58]. The relative simplicity of the RFLP profiles generated by PFGE facilitates application of the procedure in identification and epidemiological survey of bacterial pathogens [12, 70–80]. Fingerprinting, which combines PFGE with Southern transfer and hybridization, has been widely used in studying the tuberculosis nosocomial outbreak in human immunodeficiency virus (HIV)-positive populations [81–83].

RIBOTYPING

Restriction patterns can be obtained by hybridizing Southern-transferred DNA fragments with labeled bacterial ribosomal operon(s), which encode for 16S and (or) 23S rRNA. This method, called ribotyping, has been shown to have both taxonomic and epidemiological value [84, 85]. All bacteria carry these operons, which are highly conserved and therefore typeable. Particular rRNA sequences that are species- or group-specific have been also exploited in construction of oligonucleotides that have been used as probes for in situ detection of bacteria.

Ribotyping assays have been used to differentiate bacterial strains in different serotypes and to determine the serotype(s) most frequently involved in outbreaks [12, 29, 73, 79, 86–89]. This technique is especially useful

[1] Nonstandard abbreviations: RFLP, restriction fragment length polymorphism, PFGE, pulse-field gel electrophoresis, RAPD, random amplified polymorphic DNA, bDNA, branched DNA; RT, reverse transcriptase, TAS, transcription-based amplification system; TMA, transcription-mediated amplification; LCR, ligase chain reaction; SDA, strand displacement amplification; HPA, the hybridization protection assay; DELA, DNA enzyme immunoassay, SSCP, single-strand conformational polymorphisms; HCV, hepatitis C virus, HIV, human immunodeficiency virus; HPV, human papillomavirus.

in epidemiological studies for organisms with multiple ribosomal operons, such as members of the family of *Enterobacteriaceae*. Ribotyping simplifies the microrestriction patterns by rendering visible only the DNA fragments containing part or all of the ribosomal genes. The technique is less helpful when the bacterial species under investigation contains only one or a few ribosomal operons. In these instances, ribotyping typically detects only one or two bands, which limits its utility for epidemiological studies [70]. Most studies have indicated that PFGE is superior to ribotyping for analysis of common nosocomial pathogens.

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

RAPD typing, originally developed by Welsh and McClelland in 1990, involves the use of a short (usually 10 to 15 mers), arbitrarily chosen primer to amplify nearly homologous sequences of the genomic DNA under low-stringency conditions [90]. RAPD has been used to differentiate strains of various species, various serotypes within species, and various subtypes within a serotype [91–95]. It is, therefore, useful for determining whether two isolates of same species are epidemiologically related. RAPD has been used to evaluate outbreaks of infection of drug-resistant bacteria [96–98]. For potentially dangerous drug-resistant organisms such as the mycobacteria, RAPD may be a better choice than PFGE because the technique requires fewer open manipulations and the organisms are kept viable for a shorter period. RAPD is probably the simplest DNA-based subtyping method to date if a temperature-cycling instrument is available, although the usefulness for epidemiological investigations remains to be determined, particularly with regard to reproducibility concerns.

Nucleic Acid Analysis Without Amplification

NUCLEIC ACID PROBES

Nucleic acid probes are capable of identifying organisms at, above, and below the species level. The quantity of target detectable by the method depends on the size and homology of the probe chosen and the nature of the original specimen; identification of organisms in pure cultures or from isolated colonies is usually easier than detection of organism in a direct specimen. DNA probes facilitate the identification of infectious agents that do not grow rapidly. Additionally, this technique allows for the diagnosis of infections in which the organisms are not easily cultured or cannot be cultured at all. Detection of DNA with direct or culture-amplified gene probe technology has been applied to several organisms, including bacteria [14–16], viruses [17–19], mycobacteria [20–22], fungi [23, 24], and even certain parasites [25]. The technique has been also used to monitor growth as an indicator of drug resistance [99, 100] or to directly detect genes associated with antibiotic resistance [101, 102].

Gen-Probe, MicroProbe, and Digene Diagnostics are currently manufacturing several direct detection and culture identification nucleic probes that have been cleared by the US Food and Drug Administration. The procedures for the use of DNA probes are now well standardized, and the advent of synthetic short oligonucleotide DNA probes has shortened the time required for probe assay. However, direct probe techniques appear to be of limited utility owing to poor sensitivity. Nucleic acid amplification methods, described in detail below, have been explored to address this problem.

BRANCHED DNA SIGNAL AMPLIFICATION

Developed and manufactured by Chiron Corp., branched DNA (bDNA) probes are an example of signal amplification. Multiple probes as well as multiple reporter molecules are used to increase the signal in proportion to amount of target in the reaction [103, 104]. In this process, multiple specific synthetic oligonucleotides hybridize to the target and capture the target onto a solid surface. Synthetic bDNA amplifier molecules, which are enzyme-conjugated, branched oligonucleotide probes, are added. Hybridization proceeds between the amplifiers and the immobilized hybrids. After addition of a chemiluminescent substrate, light emission is measured and may be quantified [103].

In bDNA assays, all hybridization reactions occur simultaneously and the observed signal is proportional to the amount of target DNA. DNA quantification can thus be determined from a calibration curve. Because the target molecules themselves are not amplified during the process, this procedure is less likely to have contamination problems, which may be encountered with nucleic acid amplification methods. bDNA is also highly reproducible, and thus represents an excellent technological platform for monitoring therapeutic response and quantifying nucleic acids [105–109]. A separate section below deals with this particularly important issue. One of the disadvantages, however, is that the bDNA assay is generally less sensitive than enzymatic amplification techniques and usually can detect no fewer than 10^3 to 10^5 nucleic acid targets. As with many techniques, moreover, test specificities decline as greater sensitivity is sought.

Polymerase Chain Reaction

As mentioned above, for direct application to the diagnosis of infections, nucleic acid analysis without amplification often has the disadvantage of low sensitivity (high detection limits). Nucleic acid amplification techniques increase sensitivity dramatically while still retaining a high specificity. Invented by Cetus scientist Kary Mullis in 1983 [1, 2], PCR is the best-developed and most widely used method of nucleic acid amplification. An ingenious procedure, PCR is based on the ability of DNA polymerase to copy a strand of DNA by elongation of complementary strands initiated from a pair of closely spaced chemically synthesized oligonucleotide primers.

The basic technique of PCR includes repeated cycles of amplifying selected nucleic acid sequences [1, 2]. Each

cycle consists of three steps: (*a*) a DNA denaturation step, in which the double strands of the target DNA are separated; (*b*) a primer annealing step, performed at a [illegible] temperature, in which primers anneal to their complementary target sequences; and (*c*) an extension reaction step, in which DNA polymerase extends the sequences between the primers. At the end of each cycle (each consisting of the above three steps), the quantities of PCR products are theoretically doubled. The whole procedure is carried out in a programable thermal cycler. Generally, performance of 30 to 50 thermal cycles results in an exponential increase in the total number of DNA copies synthesized *[110, 111]*. Commercial systems for PCR detection of DNA targets of *Chlamydia trachomatis* and *Mycobacterium tuberculosis* are manufactured by Roche Molecular Systems *[112]*.

REVERSE TRANSCRIPTASE (RT)-PCR

Numerous modifications of the standard PCR procedure have been developed since its inception *[4, 5, 41]*. Some of these modifications effectively expand the diagnostic capabilities of PCR and have increased its utility in the clinical laboratory. RT-PCR was developed to amplify RNA targets. In this process, RNA targets are first converted to complementary DNA (cDNA) by RT, and then amplified by PCR. RT-PCR has played an important role in diagnosing RNA-containing virus infections, detecting viable *Mycobacteria* species, and monitoring the effectiveness of antimicrobial therapy *[113–115]*. The conventional reverse transcription reactions are fastidious: The enzymes cannot tolerate higher temperatures, which limits wide application of the method in clinical diagnosis. The thermostable DNA polymerase (Tth pol) and its thermostable cousins derived from other organisms have efficient reverse transcription activity and therefore can be used in detection of RNA targets without the need for a separate RT step *[116, 117]*. The higher reaction temperature increases stringency of primer hybridization and avoids the possible RNA secondary structure, so that the reaction is more specific and efficient than previous protocols that used avian myeloblastosis virus RT. Commercial kits for detection of HIV are now available that use this single enzyme technology.

NESTED PCR

Nested PCR, designed mainly to increase sensitivity (detect smaller quantities of target), uses two sets of amplification primers *[4, 118]*. One set of primers is used for the first round of amplification, which consists of 15 to 30 cycles. The amplification products of the first reaction are then subjected to a second round of amplification with another set of primers that are specific for an internal sequence that was amplified by the first primer pair *[118–120]*. Nested PCR has extremely high sensitivity because of the dual amplification process. The DNA product from the first round of amplification contains the hybridization sites for the second primer pair. The amplification by the second primer set, therefore, verifies the specificity of the first-round product. The major disadvantage of the nested-amplification protocol is the high probability of contamination during transfer of the first-round amplification products to a second reaction tube. This can be avoided either by physically separating the two amplification mixtures with a layer of wax or oil, or by designing the primer sets to utilize substantially different annealing temperatures *[4]*.

MULTIPLEX PCR

Multiplex PCR is an amplification reaction in which two or more sets of primer pairs specific for different targets are introduced in the same tube. Thus, more than one unique target DNA sequence in a specimen can be amplified at the same time *[121]*. Primers used in multiplex reactions must be carefully designed to have similar annealing temperatures, which often requires extensive empirical testing. This coamplification of multiple targets can be used for various purposes. For diagnostic uses, multiplex PCR can be set up to detect internal controls or to detect multiple pathogens from a single specimen *[115, 120, 122, 123]*. Quantitative competitive PCR, a variation of multiplex PCR, can be used to quantify the amount of target DNA or RNA in a specimen *[124, 125]*.

BROAD-RANGE PCR

Another important technical modification is the development of broad-range PCR, in which conserved sequences within phylogenetically informative genetic targets are used to diagnose microbial infection. A broad-range PCR approach has identified several novel, fastidious, or uncultivated bacterial pathogens directly from infected human tissue or blood *[126–131]*. A universal primer set designed to target herpesvirus DNA polymerases might be widely useful for diagnosing herpesvirus infection *[132]*. Broad-range rRNA PCR techniques offer the possibility of rapid bacterial identification through use of a single pair of primers targeting bacterial small-subunit (16S) rRNA or DNA *[133–136]*. The major obstacles to implementation of rapid, automated rDNA-based bacterial identification systems are background contamination and, needless to say, cost. Perkin-Elmer Applied Biosystems is developing a commercial system for broad-range bacterial amplification and sequencing.

Other Nucleic Acid Amplification Techniques

TRANSCRIPTION-BASED AMPLIFICATION SYSTEM (TAS).

Described in 1989 by Kwoh et al., TAS includes synthesis of a DNA molecule complementary to the target nucleic acid (usually RNA) and in vitro transcription with the newly synthesized cDNA as a template *[137]*. Variations on this process are referred to as **self-sustaining sequence replication ("3SR"), nucleic acid sequence-based amplification ("NASBA"), or transcription-mediated amplification (TMA)** *[138–139]*. Three enzymes, RT, RNase H, and T7 DNA-dependent RNA polymerase are used in the

reaction. Amplification steps involve the formation of cDNAs from the target RNA by using primers containing a RNA polymerase-binding site. The RNase H then degrades the initial strand of target RNA in the RNA–DNA hybrid after it has served as the template for the first primer. The second primer binds to the newly formed cDNA and is extended, resulting in the formation of double-strand cDNAs in which one or both strands are capable of serving as transcription templates for RNA polymerase. Although technically less robust and less sensitive than PCR, TMA has various merits that make it an attractive option: It works at isothermal conditions in a single tube to help minimize contamination risks *[138]*. Amplification of RNA not only makes it possible to detect RNA-containing viruses, but also lowers the detection limit for certain bacterial and fungal pathogens by using high-copy-number rRNA targets *[139]*. A commercial system for detection of *M. tuberculosis* by TMA is now available from Gen-Probe.

LIGASE CHAIN REACTION (LCR)

Also called ligase amplification reaction, LCR is a probe amplification technique first described in 1989 by Wu and Wallace *[141]*. Successful ligation relies on the contiguous positioning and correct base-pairing of the 3′ and 5′ ends of oligonucleotide probes on a target DNA molecule. In the process, oligonucleotide probes are annealed to template molecules in a head-to-tail fashion, with the 3′ end of one probe abutting the 5′ end of the second. DNA ligase then joins the adjacent 3′ and 5′ ends to form a duplicate of one strand of the target. A second primer set, complementary to the first, then uses this duplicated strand (as well as the original target) as a template for ligation Repeating the process results in a logarithmic accumulation of ligation products, which can be detected by means of the functional groups attached to the oligonucleotides *[142]*. The recently developed thermostable DNA ligase greatly simplifies this technique and has increased the specificity by helping avoid problems of blunt-end ligation at low annealing temperature *[143]*. When used after a target amplification method, such as PCR, this technique can be sensitive and is useful for the detection of point mutations. Although convenient and readily automated, one potential drawback of LCR is the difficult inactivation of the postamplification products. The nature of the technique does not allow the most widely used contamination control methods to be applied. The inclusion of a detection system within the same reaction tube would greatly decrease the possibility of contamination, which is associated with the opening of reaction tubes. A combination LCR kit for detection of both *Chlamydia trachomatis* and *Neisseria gonorrhea* is now commercially available from Abbott Labs. *[144]*.

STRAND DISPLACEMENT AMPLIFICATION (SDA)

SDA is another non-PCR nucleic acid amplification technique, developed in 1991 *[145, 146]*. In this system, DNA polymerase initiates DNA syntheses at a single-stranded nick and displaces the nicked strand during DNA synthesis. The displaced single-stranded molecule then serves as a substrate for additional simultaneous nicking and displacement reactions *[145]*. This isothermal DNA amplification procedure uses specific primers, a DNA polymerase, and a restriction endonuclease to achieve exponential amplification of target. The key technology behind SDA is the generation of site-specific nicks by the restriction endonuclease. Although complicated, SDA has two important advantages. Except for the initial denaturation step, SDA is isothermal and requires no specialized thermocycler *[146]*. In addition, SDA can be applied to either single- or double-stranded DNA.

Qβ REPLICASE SYSTEM

Initially described in 1988 *[147]*, the Qβ replicase system is based on the incorporation of a single-stranded oligonucleotide probe into an RNA molecule that can be exponentially amplified after target hybridization by the enzyme Qβ replicase *[148]*. The assay is technically straightforward. The enzyme specifically recognizes the secondary structure of the RNA from the Qβ genome, which is hybridized to the specific target. After a given probe anneals to a target, the nonhybridized material can be removed by the enzyme RNase III and subsequent wash steps. The hybridized probe is then enzymatically replicated by Qβ replicase to detectable quantities *[147, 149]*. The potential advantages associated with the Qβ replicase procedure include its remarkable speed (<30 min) and isothermal reaction conditions. The main drawback is that unbound reporter probes or nonspecifically bound reporter probes serve as templates for amplification, resulting in false-positive results. This formidable problem has been largely overcome by the use of target capture methods.

Practical information about current commercially available and Mayo Clinic-developed amplification techniques for detection of microbial pathogens are summarized in Table 1.

Analysis of Amplification Products

After target amplification, the simple or conventional version of product detection is use of agarose gel electrophoresis after ethidium bromide staining. Several other techniques have been developed not only to "visualize" the products, but to enhance both the sensitivity and specificity of amplification techniques as well. A probe-based DNA detection system has the advantage of providing sequence specificity and decreased detection limits. After routine agarose gel electrophoresis, the DNA is transferred to a solid phase, e.g., nitrocellulose or nylon membrane, and probed by a specific probe. Radiolabeled probed membranes are directly exposed to x-ray film,

whereas enzyme-labeled probed membrane may be visualized through either light or color production.

HYBRIDIZATION PROTECTION ASSAY (HPA)

HPA is a homogeneous format. The probe and the product are incubated together in a single test tube, and the binding of probe to the target is measured without further manipulation [150]. A probe labeled with an acridinium ester is added to a sample containing PCR products for identification. In a positive sample, the bound probe is protected from alkaline hydrolysis and, upon addition of peroxides, emits detectable light. The HPA does not require the binding of amplified DNA to a solid support by DNA capture or other means, can be performed in a few hours, and does not need to have excess unbound DNA probe removed [151, 152]

DNA ENZYME IMMUNOASSAY (DEIA)

DEIA is another newly developed system for detecting nucleic acid previously amplified by means of PCR [153]. An anti-dsDNA antibody exclusively recognizes the hybridization product resulting from the reaction between target DNA and a DNA probe. The final product is revealed by means of a colorimetric reaction [153]. The DEIA increases the sensitivity of a previous PCR by including enzymatic reactions. The hybridization between specific probe and PCR-amplified target DNA, as well as the formation of target DNA/probe hybrids and anti-dsDNA antibody complex, also enhances the specificity. The system is now manufactured by Sorin Biomedica Diagnostics in Europe and Incstar in the US. Variations on DEIA capture techniques have been explored recently [112, 144].

AUTOMATED DNA SEQUENCING TECHNOLOGY

Direct sequencing offers direct, rapid, and accurate analysis of amplification products. As described earlier, broad-range PCR amplifies conserved regions of a wide range of organisms [128, 133]. The amplicon sequence is first determined, then a DNA sequence-based phylogenetic analysis is performed and used to specifically identify the pathogen [154]. Current sequencing technologies include one of two approaches: electrophoretic separation, based on polyacrylamide slab gels or glass capillaries, and solid-phase sequencing, determined by matrix hybridization [128, 133].

SINGLE-STRAND CONFORMATIONAL POLYMORPHISMS (SSCP)

SSCP was first described by Orita et al. [155]. DNA is subjected to PCR with primers to a region of suspected polymorphism. The PCR products, which usually incorporate a detector marker, are examined after gel electrophoresis. Physical conformational changes in single-stranded DNA are based on the physiochemical properties of the nucleotide sequence. Variations in the physical conformation are reflected in differential gel migration. This technique is sensitive enough to detect single nucleotide substitutions. One area in which SSCP may prove to be of value is in the detection of mutations related to resistance mechanisms. SSCP, and variations on the technique, have been successfully used to examine the genes contributing to the multidrug resistance of *M. tuberculosis* [156, 157].

RFLP ANALYSIS

In postamplification RFLP analysis, the amplified DNA fragments are cut by a restriction endonuclease, separated by gel electrophoresis, and then transferred to a nitrocellulose or nylon membrane. The fragment(s) containing specific sequences may then be detected by using a labeled homologous oligonucleotide as a probe. Variations in the number and sizes of the fragments detected are referred to as RFLPs and reflect variations in both the number of loci that are homologous to the probe and the location of restriction sites within or flanking those loci [158]. An epidemiological application of RFLPs is discussed in more detail later.

Current Application of Molecular Diagnostics

CLINICAL MICROBIOLOGY

Traditionally, the clinical medical microbiology laboratory has functioned to identify the etiologic agents of infectious diseases through the direct examination and culture of clinical specimens. Direct examination is limited by the number of organisms present and by the ability of the laboratorian to successfully recognize the pathogen. Similarly, the culture of the etiologic agent depends on the ability of the microbe to propagate on artificial media and the laboratorian's choice of appropriate media for the culture. When a sample of limited volume is submitted, it is often not possible to culture for all pathogens. In such instances, close clinical correlation is essential for the judicious use of the specimen available.

Some microorganisms are either unculturable at present, extremely fastidious, or hazardous to laboratory personnel. In these instances, the diagnosis often depends on the serologic detection of a humoral response or culture in an expensive biosafety level II–IV facility. In community medical microbiology laboratories, these facilities may not be available, or it may not be economically feasible to maintain the special media required for culture of all of the rarely encountered pathogens. Thus, cultures are often sent to referral laboratories. During transit, fragile microbes may lose viability or become overgrown by contaminating organisms or competing normal flora.

The addition of molecular detection methods to the microbiology laboratory has resolved many of these problems. The exquisite sensitivity and specificity of many molecular methods allow the accurate detection of very small numbers of organisms. The direct detection of *M. tuberculosis* nucleic acid from the sputa of smear-negative patients with tuberculosis clearly illustrates this point [159–161]. The technology allows for the rapid and

Table 1. Commercially available and Mayo-developed amplification techniques for detection of microbial pathogens.

Organism detected	Manufacturer/ Institute	Basic technique adopted	Trade mark/ name	Detection system	Contamination potential	Analytical sensitivity (lower detection limit) or testing range	Clinical sensitivity %	Clinical specificity %	Primary application	Additional comments/ information
HIV-1	Roche	PCR	Amplicor®	EIA	Low to moderate	<100 copies/mL[a]	>99[a]	No claims	Confirmatory testing	Customer service 1 800-526-1247
	Roche	Quant. PCR	Monitor™	EIA	Low to moderate	400–750 000 copies/mL[b]	NA[c]	No claims	Quantitation during therapy	FDA-cleared
	Chiron	Quant. bDNA	Quantiplex™	EIA	Low	500–800 000 copies/mL[b]	NA[c]	95[b]	Quantitation during therapy	Customer service 1-800-653 1353
	Organon Teknika	NASBA	HIV-1 QL	ECL	Moderate	80 copies/mL[b]	>99[b]	>99[b]	Confirmatory testing	Customer service 1 800 682-2666
	Organon Teknika	Quant. NASBA	HIV-1 QT	ECL	Moderate	200 10^6 copies/mL[b]	NA[c]	>99[b]	Quantitation during therapy	
HCV	Roche	PCR	Amplicor®	EIA	Low to moderate	<200 copies/mL[a]	98%[a]	No claims	Confirmatory testing	
	Roche	Quant. PCR	Monitor™	EIA	Low to moderate	200-10^7 copies/mL[a]	NA[c]	No claims	Quantitation during therapy	
	Chiron	Quant. bDNA	Quantiplex™	EIA	Low	200 000–120 × 10^6 copies/mL[b]	NA[c]	98[b]	Quantitation during therapy	
	Mayo	RT-PCR		WB ECL	Moderate	<20 copies/rxn[a]	>99[a]	>99[a]	Monitoring and confirmation	Contact: 1-507-284-1441
Mycobacterium tuberculosis	Roche	PCR	Amplicor®	EIA	Low to moderate	≥20 organisms/rxn[b]	88 9–100[b]	100[b]	Smear-positive and untreated patients only	FDA-cleared. Has been used on BACTEC broth culture
	Gen-Probe	TMA	MTD	HPA-ECL	Moderate	Unknown	95.5[b]	100[b]	Smear-positive and untreated patients only	FDA-cleared Customer service: 1 800-523-5001
Chlamydia trachomatis	Roche	PCR	Amplicor®	EIA	Low to moderate	10–20 elementary bodies/rxn[b]	93 2[b]	98 4[b]	Monitoring and confirmation	FDA-cleared
	Abbott	LCR	LCX®	EIA	Moderate to high	Unknown	>95[a]	>99[a]	Monitoring and confirmation	FDA-cleared Customer service: 1-800-527-1869
	Gen-Probe	TMA	AMP	HPA ECL	Moderate	Unknown	86.7–99 2[b]	>99[b]	Monitoring and confirmation	Sensitivity varies in sexes and specimen source. FDA-cleared
Neisseria gonorrhoeae	Roche	PCR	Amplicor®	EIA	Low to moderate	No claims	No claims	No claims	Monitoring and confirmation	
	Abbott	LCR	LCX®	EIA	Moderate to high	Unknown	>95[a]	>99[a]	Monitoring and confirmation	FDA-cleared
Hepatitis B virus	Chiron	Quant. bDNA	Quantiplex™	EIA	Low	0 7–5000 × 10^6 copies/mL	NA[c]	98[b]	Quantitation and monitoring	
CMV	Roche	PCR	Amplicor®	EIA	Low to moderate	No claims	No claims	No claims	Confirmatory testing	
	Roche	Quant. PCR	Monitor™	EIA	Low to moderate	No claims	NA[c]	No claims	Quantitation and monitoring	
	Mayo	PCR		WB-ECL	Moderate	<100 CMV genome copies/rxn	70–90[a]	>95[a]	Monitoring and confirmation	
HTLV-I/II	Roche	PCR	Amplicor®	EIA	Low to moderate	No claims	No claims	No claims	Monitoring and confirmation	
Enterovirus	Roche	PCR	Amplicor®	EIA	Low to moderate	No claims	No claims	No claims	Monitoring and confirmation	
Borrelia burgdorferi	Mayo	PCR		WB-ECL	Moderate	<10 organisms	Variable[d]	>99[a]	Monitoring and confirmation	
HSV	Mayo	PCR		WB-ECL	Moderate	<100 copies/mL	70–90[a]	>99[a]	Monitoring and confirmation	
Bordetella pertussis	Mayo	PCR		WB ECL	Moderate	<100 organisms/mL	>95[a]	95[a]	Monitoring and confirmation	

(continued)

Table 1. Continued

Organism detected	Manufacturer/ Institute	Basic technique adopted	Trade mark/ name	Detection system	Contamination potential	Analytical sensitivity (lower detection limit) or testing range	Clinical sensitivity %	Clinical specificity	Primary application	Additional comments/ information
JC virus	Mayo	PCR		WB-ECL	Moderate	<100 copies/mL	75[a]	>95[a]	Monitoring and confirmation	
Babesia microti	Mayo	PCR		WB ECL	Moderate	<100 copies/mL	>95[a]	>99[a]	Monitoring and confirmation	
HGE	Mayo	PCR		WB-ECL	Moderate	<100 copies/mL	>95[a]	>99[a]	Monitoring and confirmation	
Tropheryma whippelii	Mayo	PCR		WB ECL	Moderate	<100 copies/mL	>95[a]	>99[a]	Monitoring and confirmation	More sensitive than histology for diagnosis
Epstein–Barr virus	Mayo	PCR		WB-ECL	Moderate	<100 copies/mL	Unknown[a]	>95[a]	Monitoring and confirmation	
Varicella-zoster virus	Mayo	PCR		WB ECL	Moderate	<100 copies/mL	Unknown[a]	>95[a]	Monitoring and confirmation	

NASBA, nucleic acid sequence based amplification; WB, Western blot; EIA, enzyme immunoassay; ECL, electrochemiluminescence; HTLV, human T cell lymphotropic virus; CMV, cytomegalovirus; HGE, human granulocytic ehrlichiosis; NA, not applicable; rxn, reaction.

[a] Based on Mayo's experience.
[b] Based on manufacturer's claim.
[c] For testing of seropositive patients only.
[d] 95% on synovial fluid specimens, 30% on cerebrospinal fluid and blood specimens from acute cases
[e] Too few cases to evaluate sensitivity.

accurate identification of the etiologic agent in a time substantially shorter than traditional methods. This allows for earlier initiation of a focused antimicrobial regimen and decreases the likelihood of disease progression.

In selected situations, the limitations imposed by the ability of an organism to be cultured and the selection of appropriate media and culture conditions may be replaced by the use of molecular microbiology. Microbial DNA/RNA extracted from a clinical specimen may be analyzed for the presence of various organism-specific nucleic acid sequences regardless of the physiological requirements or viability of the organism *[136, 162–165]*. For example, the inability to culture and analyze the principal etiologic agent of non-A, non-B hepatitis limited medical advances in this area. Using various molecular methods, however, investigators have been able to isolate hepatitis C virus (HCV) nucleic acid *[166]*. Analysis and cloning of the HCV genome has provided the viral antigens necessary for the development of specific serologic tests *[167–169]*. Currently, RT-PCR allows for the identification, quantification, and sequence analysis of the HCV genome in infected individuals *[117, 170, 171]*.

Another unculturable microbe that has been specifically detected by PCR and probe analysis is *Tropheryma whippelii*, the causative agent of Whipple disease *[128, 172, 173]*. Because of the inability of this organism to grow on conventional media and the lack of a serologic test, diagnosis of Whipple disease is usually based on clinical and specific biopsy findings. Patients with Whipple disease often have gastrointestinal manifestations and undergo endoscopy. Small bowel biopsies reveal foamy histiocytes filling the lamina propria. The definitive diagnosis is made with the identification of non-acid-fast, periodic acid-shift-positive, diastase-resistant bacillary forms within the histiocytes. Extraintestinal Whipple disease, principally seen as arthritis and central nervous system involvement, may be missed entirely unless the clinician and pathologist have a high index of suspicion. Even so, the diagnosis in such instances may prove difficult. Advances in the molecular detection of *T. whippelii* have resolved this dilemma *[128, 172, 173]*. On the basis of bacterial 16S rRNA gene sequence analysis, an emerging pathogen, *Bordetella holmesii*, has been successfully identified in the immunocompromised hosts *[130, 131]*. Additionally, the DNA from a single clinical specimen, such as a knee fluid aspirate, may be tested for several etiologic agents in a differential diagnosis. In such instances, the specimen may also be analyzed for other fastidious and difficult-to-culture agents of infectious arthritis, such as *N. gonorrhea* or *Borrelia burgdorferi [14, 15, 60, 103, 125, 174]*.

As alluded to earlier, molecular methods may also be useful in instances of limited specimen volume *[175, 176]*. Even in low-volume specimens, enough DNA/RNA can often be extracted to allow performance of numerous molecular assays. However, though molecular methods are very sensitive, we emphasize that, like culture and direct

examination, clinically relevant results are ultimately reliant on the submission of quality specimens [177–178].

Some organisms, although not difficult to culture, are encountered infrequently and require special media for isolation. In these instances, culturing may not be cost-effective for smaller laboratories because the reagents may expire before usage; these samples may also be sent to reference laboratories for culturing, for the sake of economy. Again, fragile organisms may die in transit or become overgrown by contaminating bacteria, thereby making the subsequent culture useless. If molecular microbiology facilities are not available in community laboratories, nucleic acids extracted by the use of commercially available kits may be sent frozen to molecular reference facilities. Alternatively, if molecular facilities are available, PCR primers and probes for relatively rare microorganisms may be maintained frozen at −70 °C for extended periods and used when needed. This may eliminate the need for special culture media and circumvent problems related to specimen transit. As molecular techniques become more widely available, the spectrum of rapid and cost-effective clinical microbiology testing available to smaller laboratories can be extended.

Molecular methods of detection may also play a role in laboratory safety. Organisms such as *Coxiella burnetti*, *M. tuberculosis*, *Coccidioides immitis*, and several viruses causing severe hemorrhagic fevers are laboratory hazards [179–182]. These organisms are easily cultured, but may infect laboratory personnel and cause serious illness or death. The handling of these organisms requires specially trained personnel, special equipment, and expensive ventilated facilities—all of which increase laboratory costs. Molecular methods may be used to detect such organisms directly from clinical specimens, without exposing laboratory personnel to biologically amplified organisms. After the initial extraction procedure, only noninfectious materials are handled.

The molecular detection of microbes with a known susceptibility profile is an effective replacement of the traditional culture. An excellent example is the molecular detection of *Bordetella pertussis* [176]. This organism is a relatively slow grower, requires specially supplemented and more costly media, and has a known susceptibility profile. The molecular detection of *Bordetella pertussis* can save time, lower laboratory costs with regard to special media, and allow for the more rapid initiation of effective therapy [176]. If variable antimicrobial susceptibility profiles exist, culture for susceptibility testing is still necessary. Molecular methods for the detection of antimicrobial-resistant strains are in development and in the future may replace traditional susceptibility testing (see below). Until then, molecular screening may be used to determine which patients should be cultured for subsequent susceptibility testing.

In recent years, the demand for quantification of nucleic acid targets has been growing [183, 184]. By use of molecular methods, the microbial load of an infecting pathogen may be determined and its genotype may also be evaluated. Viral load data are used to monitor therapeutic responsiveness and may yield prognostic information regarding the progression of disease. Until recently, however, the task of quantitative nucleic acid amplification has been very difficult to accomplish. Because the amplification techniques yielded products in an exponential manner until a plateau was reached, any factor interfering with the exponential nature of the amplification process would therefore affect the result of the quantitative assay. In practice, many factors can affect the efficiency of the PCR reaction throughout the amplification procedures and result in the differences between theoretical and actual yields of the reaction. Now, however, kit-based technologies make it possible for many laboratories to carry out quantitative determinations.

Viral load determinations are currently used for evaluating HIV and HCV disease by the use of PCR and bDNA technology [185–187]. When used with other surrogate markers such as CD4 cell count, determination of plasma HIV viral load is an early and accurate marker of disease progression [188–191]. This may result in better predictors of disease progression and outcome, as well as criteria for initiation and modification of antiviral therapy.

CLINICAL EPIDEMIOLOGY AND INFECTION CONTROL

The investigation and control of nosocomial infections is a complex issue that involves clinical, infection-control, and laboratory personnel. The efforts of both the microbiologist and the hospital epidemiologist are facilitated greatly by the availability of the newer molecular epidemiological typing techniques. Molecular diagnostic techniques have been successfully used in the investigation and control of classical and emerging nosocomial pathogens, such as the enterobacteriaceae, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, coagulase-negative staphylococci, enterococci, *Candida albicans*, *M. tuberculosis*, and *Chlamydia pneumoniae* [79, 192–194]. Application of DNA probe-based assays allows the diagnosis of other nosocomial infections caused by respiratory syncytial virus [195], varicella-zoster virus, herpes simplex virus [196], and legionella [197] to be made in only a few hours. The molecular techniques have played an important role in the detection, identification, and antimicrobial susceptibility testing of many nosocomial pathogens [83, 96, 97, 198]. A good example is the use of PCR-RFLP analysis in combination with Southern transfer and hybridization (fingerprinting) to study the multiple drug-resistant *M. tuberculosis* nosocomial outbreak in HIV-positive groups in Miami [81] and New York [82, 83].

The ability to rapidly and unambiguously characterize organisms suspected of causing a disease outbreak is critical to public health and hospital infection-control endeavors. Recent contributions to clinical and hospital epidemiology have depended on PCR. Several putative outbreaks of infections have been investigated by molec-

ular techniques. Such examples include investigation of several temporally clustered cases of *Streptococcus pyogenes* invasive disease in Air Force recruits [199], a case cluster of lymphogranuloma venereum caused by *Chlamydia trachomatis* serovar L1 in homosexual men [200], and an outbreak of *E. coli* O157:H7 infection from contaminated deer jerky [80].

Significantly, a PCR analysis was recently successfully used to identify the hantavirus agent responsible for an outbreak of fatal infections in the US Southwest. In May 1993, a mysterious respiratory illness outbreak was reported in the Four Corners region, which includes New Mexico, Arizona, Colorado, and Utah. Patients were defined as having unexplained adult respiratory distress syndrome or acute bilateral pulmonary interstitial infiltrates. Mortality in confirmed patients was >75%. Preliminary serologic studies found antibodies in patients' sera in patterns suggesting cross-reactivity (but not identity) with previously known hantaviruses [180]. By comparing genome sequences of available hantavirus strains, precise regions of sequence conservation within the G2 protein coding region of the M segment of the hantavirus genome were identified [201, 202]. Deoxyoligonucleotide primers were designed for detection of small amounts of hantavirus genome by a nested RT-PCR assay. The genetic detection assay amplified hantavirus-specific DNA fragments from RNA extracted from the tissues of patients and deer mice caught at or near patients' residences, revealing the associated virus to be a new hantavirus and providing a direct genetic link between infection in patients and rodents [203].

Molecular techniques are being used increasingly in epidemiological and clinical investigations. Among viral infections, the human papillomavirus (HPV) is a common cause of dysplasia, intraepithelial neoplasia, and carcinoma in the female genital tract. Certain types, such as types 16 and 18, have been regarded as high-risk cancer-associated HPVs, whereas types 6 and 11 are regarded as low-risk HPVs [204, 205]. Use of DNA hybridization assays in cervical swabs or fresh cervical biopsy specimens to determine HPV infection and viral types has provided helpful information for clinical assessment and treatment of patients [206, 207]. In HCV infections, different genotypes have been reported to alter disease severity, change treatment response, and influence virus–host interactions [208]. A specific primer set to the 5'-untranslated region has been designed to allow detection of HCV nucleic acids of different genotypes [209]. By using PCR followed by automated direct sequencing, several studies have revealed that the most common genotypes of HCV in the US and Western Europe are 1a and 1b; other genotypes, including 2a, 2b, 3, 4, 5, and 6, have their own distinct global distributions [210, 211]. A new PCR-based rHCV genotyping system has been recently developed to identify HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a; it may be useful for a large-scale determination of HCV genotypes in clinical studies [212].

Molecular techniques have been used to directly detect resistance genes or mutations that result in resistance in organisms. The *mecA* gene that codes for resistance to methicillin in Staphylococci has been detected by PCR, multiplex PCR, and bDNA assays [123, 213, 214]. Defining the mutations responsible for resistance to microbial agents has led to new methods for monitoring efficacy of antimicrobial therapy. Successful investigations have been carried out on both bacterial and viral resistance mechanisms. A PCR assay has been used to detect mutations in the *rpoB* locus associated with rifampin resistance in *M. tuberculosis* [157, 159, 215]. The previously discussed TMA technique has been described for detection of the point mutations resulting in zidovudine resistance in stains of HIV [140]. Determination of the structural basis of resistance of HIV to viral polymerase inhibitors has been described in detail elsewhere [106, 216, 217]. Another example is the finding that certain point mutations in the herpes simplex virus-encoded thymidine kinase gene are responsible for the occurrence of acyclovir resistance [218]. Determining acyclovir resistance by detecting these point mutations is extremely important in patients undergoing long-term therapy and in patients with AIDS or other immunosuppressed states [156, 219, 220].

Future Applications

Molecular screening of particular at-risk populations for a group of possible pathogens is an exciting area of development in molecular microbiology. For example, numerous etiologic agents cause debilitating gastroenteritis in immunosuppressed patient populations, including mycobacteria (i.e., *M. avium* complex and *M. genevense*), parasites (i.e., *Cryptosporidum, Microsporidum*), viruses (i.e., rotovirus, Norwalk agent), and typical bacterial pathogens (*E coli* variants, *Salmonella, Shigella,* and *Campylobacter*). Traditionally, different methods of detection are used for each group of intestinal pathogens. This requires special media, equipment, and expensive facilities for the culture of mycobacteria; expertise in the identification of parasites in ova and parasite stool preparations; virology facilities; and special media for the workup of bacterial enteric pathogens. Although these tests may be relatively inexpensive individually, an adequate workup for enteric pathogens can be quite costly.

Molecular techniques exist and are being developed that may be used to screen individuals within a particular patient population for the most probable etiologic agents of disease. Nucleic acids extracted from the stool of patients with gastroenteritis may be examined with organism- or group-specific nucleic acid primers and probes. In this manner, one single test may be used to single out the etiologic agent of disease among numerous possibilities.

The techniques being used for molecular screening include the newer nucleic acid "chip" technologies, multiplex PCR, and the use of broad-range PCR primers and subsequent nucleic acid sequence analysis. "DNA chips,"

developed and manufactured by several companies, are basically the product of bonding or direct synthesis of numerous specific DNA probes on a stationary, often silicon-based support *[221–225]*. Within the particular well, hybridization reactions occur if the appropriate sequence or probe "feature" is present in the DNA or RNA analyte. Because numerous features are present on a single chip, several microbial pathogens or targets may be detected in one test. The chip may be tailored to particular disease processes. This technology is easily performed and readily automated.

Similarly, multiplex PCR utilizes numerous primers within a single reaction tube so as to amplify nucleic acid fragments from different targets. Specific nucleic acid amplification should occur if the appropriate target DNA is present in the sample tested *[115, 120, 122, 123]*. Detection may then be accomplished by traditional Southern transfer and subsequent nucleic acid probe, by enzyme immunoassay methods, or by "gene-chip" analysis. This technology is limited by the number of primers that can be included in a single reaction, primer–primer interference, and nonspecific nucleic acid amplification.

Finally, several pathogens within taxonomically related groups may be screened with broad-range PCR primers and detected by nucleic acid sequence or probe analysis *[126–128, 226]*. Primers are chosen on the basis of nucleic acid sequence comparisons to include pathogenic agents and, if possible, to exclude possible environmental contaminants. For example, the use of broad-range PCR primers and sequence analysis has successfully detected diseases caused by members of the *Rickettsiaceae*; in particular, the agents of ehrlichiosis have been identified and speciated *[154]*. This technique is quite useful in instances in which the differential diagnosis can be limited to a particular group of organisms.

Future applications in the field of molecular microbiology include the rapid detection of microbial resistance and, we hope, with the development of more user-friendly systems, the expansion of these technologies to smaller institutions and hospitals. The use of these biochemical methods and reactions in the specific identification of infectious agents at the nucleic acid level truly represents a synthesis of the clinical chemistry and clinical microbiology laboratories.

References

1. Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed reaction. Methods Enzymol 1987;155: 335–50.
2. Mullis KB. The unusual origin of the polymerase chain reaction. Sci Am 1990;262:56–65.
3. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988;239:487–91.
4. Erlich HA, Gelfand D, Sninsky JJ. Recent advances in the polymerase chain reaction [Review]. Science 1991;252:1643–51.
5. Persing DH. Polymerase chain reaction, trenches to benches [Review] J Clin Microbiol 1991;29:1281–5.
6. Thurm V, Gericke B. Identification of infant food as a vehicle in a nosocomial outbreak of *Citrobacter freundii*, epidemiological subtyping by allozyme, whole-cell protein, and antibiotic resistance. J Appl Bacteriol 1994;76:553–8.
7. Pierson CL, Friedman BA. Comparison of susceptibility to beta-lactam antimicrobial agents among bacteria isolated from intensive care units Diag Microbiol Infect Dis 1992;15:19S–30S
8. Lin D, Lehmann PF, Hamory BH, Padhye AA, Durry E, Pinner RW, Lasker BA. Comparison of three typing methods for clinical and environmental isolates of *Aspergillus fumigatus*. J Clin Microbiol 1995;33:1596–601.
9. Stoakes L, John MA, Lannigan R, Schieven BC, Ramos M, Harley D, Hussain Z. Gas–liquid chromatography of cellular fatty acids for identification of staphylococci. J Clin Microbiol 1994,32 1908–10.
10. Blanc DS, Lugeon C, Wenger A, Siegrist HH, Francioli P. Quantitative antibiogram typing using inhibition zone diameters compared with ribotyping for epidemiological typing of methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol 1994,32: 2505–9.
11. Bernstein JM, Sagahtaheri-Altaie S, Dryja DM, Wactawski-Wende J. Bacterial interference in nasopharyngeal bacterial flora of otitis-prone and non-otitis-prone children. Acta Oto-Rhino-Laryn Belg 1994;48:1–9.
12. Tenover FC, Arbeit R, Archer G, Biddle J, Byrne S, Goering R, et al. Comparison of traditional and molecular methods of typing isolates of *Staphylococcus aureus*. J Clin Microbiol 1994,32: 407–15.
13. Asai S, Noda M, Yamamura M, Hozumi Y, Takase I, Nitta H, et al. Comparative study of the cellular fatty acids of methicillin-resistant and susceptible *Staphylococcus aureus*. APMIS 1993; 101:753–61.
14. Granato PA, Franz MR. Use of the Gen-Probe PACE system for the detection of *Neisseria gonorrhoeae* in urogenital samples. Diag Microbiol Infect Dis 1990;3:217–21.
15. Lewis JS, Fakile O, Foss E, Legarza G, Leskys A, Lowe K, Powning D. Direct DNA probe assay for *Neisseria gonorrhoeae* in pharyngeal and rectal specimens. J Clin Microbiol 1993;31:2783–5.
16. Daly JA, Clifton NL, Seskin KC, Gooch WM III. Use of rapid, nonradioactive DNA probes in culture confirmation tests to detect *Streptococcus agalactiae*, *Haemophilus influenzae*, and *Enterococcus* spp from pediatric patients with significant infections. J Clin Microbiol 1991;29:80–2.
17. Clarke LM, Sierra MF, Daidone BJ, Lopez N, Covino JM, McCormack WM. Comparison of the Syva MicroTrak enzyme immunoassay and Gen-Probe PACE 2 with cell culture for diagnosis of cervical *Chlamydia trachomatis* infection in a high-prevalence female population. J Clin Microbiol 1993,31:968–71.
18. Denniston KJ, Hoyer BH, Smedile A, Wells FV, Nelson J, Gerin JL. Cloned fragment of the hepatitis delta virus RNA genome: sequence and diagnostic application. Science 1986;232: 873–5.
19. Kiviat NB, Koutsky LA, Critchlow CW, Galloway DA, Vernon DA, Peterson ML, et al. Comparison of Southern transfer hybridization and dot filter hybridization for detection of cervical human papillomavirus infection with types 6, 11, 16, 18, 31, 33, and 35. Am J Clin Pathol 1990,94:561–5.
20. Drake TA, Hindler JA, Berlin OG, Bruckner DA. Rapid identification of *Mycobacterium avium* complex in culture using DNA probes. J Clin Microbiol 1987;25:1442–5.
21. Gonzalez R, Hanna BA. Evaluation of Gen-Probe DNA hybridization systems for the identification of *Mycobacterium tuberculosis*

and *Mycobacterium avium-intracellulare* Diag Microbiol Infect Dis 1987;8:69–77.
22. Musial CE, Tice LS, Stockman L, Roberts GD. Identification of mycobacteria from culture by using the Gen-Probe rapid diagnostic system for *Mycobacterium avium* complex and *Mycobacterium tuberculosis* complex. J Clin Microbiol 1988;26:2120–3.
23. Hall GS, Pratt-Rippin K, Washington JA. Evaluation of a chemiluminescent probe assay for identification of *Histoplasma capsulatum* isolates. J Clin Microbiol 1992;30:3003–4.
24. Huffnagle KE, Gander RM. Evaluation of Gen-Probe's *Histoplasma capsulatum* and *Cryptococcus neoformans* Accu-Probes. J Clin Microbiol 1993;31:419–21.
25. Romero JL, Descoteaux S, Reed S, Orozco E, Santos J, Samuelson J. Use of polymerase chain reaction and nonradioactive DNA probes to diagnose *Entamoeba histolytica* in clinical samples. Arch Med Res 1992;23:277–9.
26. Mayer LW. Use of plasmid profiles in epidemiologic surveillance of disease outbreaks and in tracing the transmission of antibiotic resistance. Clin Microbiol Rev 1988;1:228–43
27. Wachsmuth K. Genotypic approaches to the diagnosis of bacterial infections: plasmid analyses and gene probes Infect Control 1985;6:100–9.
28. Sabria-Leal M, Morthland VH, Pedro-Botet ML, Sopena N, Gimenez-Perez M, Branchini ML, Pfaller MA. Molecular epidemiology for local outbreaks of methicillin resistant *Staphylococcus aureus* (MRSA). The need for several methods. Eur J Epidemiol 1994;10:325–30.
29. Grattard F, Pozzetto B, Berthelot P, Rayet I, Ros A, Lauras B, Gaudin OG. Arbitrarily primed PCR, ribotyping, and plasmid pattern analysis applied to investigation of a nosocomial outbreak due to *Enterobacter cloacae* in a neonatal intensive care unit. J Clin Microbiol 1994;32:596–602.
30. Wu SX, Liu YX. Molecular epidemiologic study of burn wound infection caused by *Staphylococcus aureus* in children. Chin Med J 1994;107:570–3.
31. Pitt TL, Gaston MA. Bacteriophage typing. Methods Mol Biol 1995;46:15–26.
32. Martin de Nicolas MM, Vindel A, Offez-Nieto JA. Epidemiological typing of clinically significant strains of coagulase-negative staphylococci. J Hosp Infect 1995;29:35–43.
33. Usera MA, Popovic T, Bopp CA, Strockbine NA. Molecular subtyping of *Salmonella ententidis* phage type 8 strains from the United States. J Clin Microbiol 1994;32:194–8.
34. Sader HS, Hollis RJ, Pfaller MA. The use of molecular techniques in the epidemiology and control of infectious diseases [Review]. Clin Lab Med 1995;15:407–31.
35. Choi YJ, Hu Y, Mahmood A. Clinical significance of a polymerase chain reaction assay for the detection of *Mycobacterium tuberculosis*. Am J Clin Pathol 1996;105:200–4.
36. Catry MA, Borrego MJ, Cardoso J, Azevedo J, Santo I. Comparison of the Amplicor *Chlamydia trachomatis* test and cell culture for the detection of urogenital chlamydial infections. Genitourin Med 1995;71:247–50.
37. Cherubin CE. Antibiotic resistance of *Salmonella* in Europe and the United States. Rev Infect Dis 1981;3:1105–26.
38. Cohen ML, Tauxe RV. Drug-resistant Salmonella in the United States: an epidemiologic perspective. Science 1986;234: 964–9.
39. Sahm DF, O'Brien TF. Detection and surveillance of antimicrobial resistance [Review]. Trends Microbiol 1994;2:366–71.
40. Livornese LL Jr, Dias S, Samel C, Romanowski B, Taylor S, May P, et al. Hospital-acquired infection with vancomycin-resistant *Enterococcus faecium* transmitted by electronic thermometers. Ann Intern Med 1992;117:112–6.
41. Wagar EA. Direct hybridization and amplification applications for the diagnosis of infectious diseases [Review] J Clin Lab Anal 1996;10:312–25
42. Cormican MG, Glennon M, Riain UN, Smith T, Flynn J, Gannon F Evaluation of a PCR assay for detection of *Mycobacterium tuberculosis* in clinical specimens. Diag Microbiol Infect Dis 1995;22:357–60
43. Beavis KG, Lichty MB, Jungkind DL, Giger O. Evaluation of Amplicor PCR for direct detection of *Mycobacterium tuberculosis* from sputum specimens. J Clin Microbiol 1995;33:2582–6.
44. Enroth H, Engstrand L. Immunomagnetic separation and PCR for detection of *Helicobacter pylori* in water and stool specimens. J Clin Microbiol 1995;33:2162–5.
45. Mathis A, Deplazes P. PCR and in vitro cultivation for detection of *Leishmania* spp. in diagnostic samples from humans and dogs. J Clin Microbiol 1995;33:1145–9.
46. Lin JJ, Harn HJ, Hsu YD, Tsao WL, Lee HS, Lee WH. Rapid diagnosis of tuberculous meningitis by polymerase chain reaction assay of cerebrospinal fluid. J Neurol 1995;242:147–52.
47. Williams WJ, Radulovic S, Dasch GA, Lindstrom J, Kelly DJ, Oster CN, Walker DH. Identification of *Rickettsia conorii* infection by polymerase reaction in a soldier returning from Somalia Clin Infect Dis 1994;19:93–9.
48. Elvin K. Laboratory diagnosis and occurrence of *Pneumocystis carinii*. Scand J Infect Dis Suppl 1994;94:1–34.
49. Yamashita Y, Kohno S, Koga H, Tomono K, Kaku M. Detection of *Bacteroides fragilis* in clinical specimens by PCR. J Clin Microbiol 1994;32:679–83.
50. Miller N, Hernandez SG, Cleary TJ. Evaluation of Gen-Probe amplified *Mycobacterium tuberculosis* direct test and PCR for direct detection of *Mycobacterium tuberculosis* in clinical specimens. J Clin Microbiol 1994;32:393–7.
51. Busch MP, Wilber JC, Johnson P, Tobler L, Evans CS. Impact of specimen handling and storage on detection of hepatitis C virus RNA. Transfusion 1992;32:420–5.
52. Greer CE, Peterson SL, Kiviat NB, Manos MM. PCR amplification from paraffin-embedded tissues, effects of fixative and fixation time. Am J Clin Pathol 1991;95:117–24.
53. Kilian M, Sorensen I, Frederiksen W. Biochemical characteristics of 130 recent isolates from *Haemophilus influenzae* meningitis. J Clin Microbiol 1979;9 409–12.
54. Maslow JN, Brecher SM, Adams KS, Durbin A, Loring S, Arbeit RD. Relationship between indole production and differentiation of *Klebsiella* species: indole-positive and -negative isolates of *Klebsiella* determined to be clonal. J Clin Microbiol 1993;31: 2000–3.
55. Dijkshoorn L, Aucken H, Gerner-Smidt P, Janssen P, Kaufmann ME, Garaizar J, et al. Comparison of outbreak and nonoutbreak *Acinetobacter baumannii* strains by genotypic and phenotypic methods. J Clin Microbiol 1996;34:1519–25.
56. Maki DG, Rhame FS, Mackel DC, Bennett JV. Nationwide epidemic of septicemia caused by contaminated intravenous products. I. Epidemiologic and clinical features. Am J Med 1976;60: 471–85.
57. Tenover FC, McGowan JE Jr. Reasons for the emergence of antibiotic resistance [Review]. Am J Med Sci 1996;311:9–16.
58. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 1995;33:2233–9.
59. Goswitz JJ, Willard KE, Fasching CE, Peterson LR. Detection of *gyrA* gene mutations associated with ciprofloxacin resistance in methicillin-resistant *Staphylococcus aureus*: analysis by polymerase chain reaction and automated direct DNA sequencing. Antimicrobiol Agents Chemother 1992;36:1166–9.
60. Ng LK, Carballo M, Dillon JA. Differentiation of *Neisseria gonor-*

rhoeae isolates requiring proline, citrulline, and uracil by plasmid content, serotyping, and pulsed-field gel electrophoresis. J Clin Microbiol 1995;33:1039–41.

61. Mulligan ME, Kwok RY, Citron DM, John JF Jr, Smith PB. Immunoblots, antimicrobial resistance, and bacteriophage typing of oxacillin-resistant *Staphylococcus aureus*. J Clin Microbiol 1988;26:2395–401.
62. Bannerman TL, Hancock GA, Tenover FC, Miller JM. Pulsed-field gel electrophoresis as a replacement for bacteriophage typing of *Staphylococcus aureus*. J Clin Microbiol 1995;33:551–5.
63. Selander RK, Caugant DA, Ochman H, Musser JM, Gilmour MN, Whittam TS. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics [Review]. Appl Environ Microbiol 1986;51:873–84.
64. Blair JE, Williams RE. Phage typing of staphylococci [Review] Bull WHO 1961;24:771–84.
65. Hickman-Brenner FW, Stubbs AD, Farmer JJ III. Phage typing of *Salmonella enteritidis* in the United States. J Clin Microbiol 1991;29:2817–23
66. Morosini MI, Canton R, Martinez-Beltran J, Negri MC, Perez-Diaz JC, Baquero F, Blazquez J. New extended-spectrum TEM-type beta-lactamase from *Salmonella enterica* subsp. *enterica* isolated in a nosocomial outbreak Antimicrob Agents Chemother 1995;39:458–61.
67. Anderson JR, Smith MD, Kibbler CC, Holton J, Scott GM. A nosocomial outbreak due to non-encapsulated *Haemophilus influenzae*: analysis of plasmids coding for antibiotic resistance. J Hosp Infect 1994;27:17–27.
68. Mickelsen PA, Plorde JJ, Gordon KP, Hargiss C, McClure J, Schoenknecht FD, et al. Instability of antibiotic resistance in a strain of *Staphylococcus epidermidis* isolated from an outbreak of prosthetic valve endocarditis. J Infect Dis 1985;152:50–8.
69. Schwartz DC, Cantor CR. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. Cell 1984;37:67–75.
70. Arbeit RD, Slutsky A, Barber TW, Maslow JN, Niemczyk S, Falkinham JO, et al. Genetic diversity among strains of *Mycobacterium avium* causing monoclonal and polyclonal bacteremia in patients with AIDS. J Infect Dis 1993;167:1384–90.
71. Murray BE, Singh KV, Heath JD, Sharma BR, Weinstock GM. Comparison of genomic DNAs of different enterococcal isolates using restriction endonucleases with infrequent recognition sites. J Clin Microbiol 1991;28:2059–63.
72. Prevost G, Jaulhac B, Piemont Y. DNA fingerprinting by pulsed-field gel electrophoresis is more effective than ribotyping in distinguishing among methicillin-resistant *Staphylococcus aureus* isolates. J Clin Microbiol 1992;30:967–73.
73. Kristjansson M, Samore MH, Gerding DN, DeGirolami PC, Bettin KM, Karchmer AW, Arbeit RD. Comparison of restriction endonuclease analysis, ribotyping, and pulsed-field gel electrophoresis for molecular differentiation of *Clostridium difficile* strains. J Clin Microbiol 1994;32:1963–9.
74. Back NA, Linnemann CC Jr, Pfaller MA, Staneck JL, Morthland V. Recurrent epidemics caused by a single strain of erythromycin-resistant *Staphylococcus aureus*. The importance of molecular epidemiology. JAMA 1993;270:1329–33.
75. Brosch R, Buchrieser C, Rocourt J. Subtyping of *Listeria monocytogenes* serovar 4b by use of low-frequency-cleavage restriction endonucleases and pulsed-field gel electrophoresis. Res Microbiol 1991;142:667–75.
76. Samore MH, Venkataraman L, DeGirolami PC, Arbeit RD, Karchmer AW. Clinical and molecular epidemiology of sporadic and clustered cases of nosocomial *Clostridium difficile* diarrhea. Am J Med 1996;100:32–40.
77. Banatvala N, Magnano AR, Cartter ML, Barrett TJ, Bibb WF, Vasile LL, et al Meat grinders and molecular epidemiology: two supermarket outbreaks of *Escherichia coli* O157:H7 infection. J Infect Dis 1996;173:480–3.
78. Layton MC, Hierholzer WJ Jr, Patterson JE The evolving epidemiology of methicillin-resistant *Staphylococcus aureus* at a university hospital. Infect Control Hosp Epidemiol 1995;16:12–7.
79. Van Belkum A, Van Leeuwen W, Verkooyen R, Can Sacilick S, Cokmus C, Verbrugh H Dissemination of a single clone of methicillin-resistant *Staphylococcus aureus* among Turkish hospitals J Clin Microbiol 1997;35:978–81
80. Keene WE, Sazie E, Kok J, Rice DH, Hancock DD, Balan VK, et al. An outbreak of *Escherichia coli* O157:H7 infections traced to jerky made from deer meat. JAMA 1997;277:1229–31.
81. Beck-Sague C, Dooley SW, Hutton MD, Otten J, Breeden A, Crawford JT, et al. Hospital outbreak of multidrug-resistant *Mycobacterium tuberculosis* infections factors in transmission to staff and HIV-infected patients. JAMA 1992;268 1280–6.
82. Dooley SM, Jarvis WR, Marlone WJ, Snider DE. Multidrug-resistant *Mycobacterium tuberculosis*. Ann Intern Med 1992;117:257–9.
83. Pearson ML, Jereb JA, Frieden TR, Crawford JT, Davis BJ, Dooley SW, Farvis WR. Nosocomial transmission of multidrug-resistant *Mycobacterium tuberculosis*. Ann Intern Med 1992;117:191–6.
84. Grimont F, Grimont PA. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. Ann Inst Past Microbiol 1986;37B:165–75.
85. Altwegg M, Mayer LW. Bacterial molecular epidemiology based on a nonradioactive probe complementary to ribosomal RNA [Review] Res Microbiol 1989;140:325–33.
86. Moyer NP, Luccini GM, Holcomb LA, Hall NH, Altwegg M. Application of ribotyping for differentiating aeromonads isolated from clinical and environmental sources. Appl Environ Microbiol 1992;58:1940–4.
87. Baloga AO, Harlander SK. Comparison of methods for discrimination between strains of *Listeria monocytogenes* from epidemiological surveys. Appl Environ Microbiol 1991;57:2324–31.
88. Arthur M, Arbeit RD, Kim C, Beltran P, Crowe H, Steinbach S, et al. Restriction fragment length polymorphisms among uropathogenic *Escherichia coli* isolates: pap-related sequences compared with *rrn* operons. Infect Immun 1990;58:471–9
89. Okwumabua O, Staats J, Chengappa MM. Detection of genomic heterogeneity in *Streptococcus suis* isolates by DNA restriction fragment length polymorphisms of rRNA genes (ribotyping). J Clin Microbiol 1995;33:968–72.
90. Welsh J, McClelland M. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 1990;18:7213–8.
91. Mazurier S, van de Giessen A, Heuvelman K, Wernars K. RAPD analysis of *Campylobacter* isolates: DNA fingerprinting without the need to purify DNA Lett Appl Microbiol 1992;14:260–2
92. McMillin DE, Muldrow LL. Typing of toxic strains of *Clostridium difficile* using DNA fingerprints generated with arbitrary polymerase chain reaction primers. FEMS Microbiol Lett 1992;71:5–9.
93. MacGowan AP, O'Donaghue K, Nicholls S, McLauchlin J, Bennett PM, Reeves DS. Typing of *Listeria* spp. by random amplified polymorphic DNA (RAPD) analysis. J Clin Microbiol 1993;38:322–7.
94. Goyal M, Young D, Zhang Y, Jenkins PA, Shaw RJ. PCR amplification of variable sequence upstream of *katG* gene to subdivide strains of *Mycobacterium tuberculosis* complex. J Clin Microbiol 1994;32:3070–1.
95. Sandery M, Coble J, McKersie-Donnolley S. Random amplified polymorphic DNA (RAPD) profiling of *Legionella pneumophila*. Lett Appl Microbiol 1994;19:184–7.
96. Elaichouni A, Verschraegen G, Claeys G, Devleeschouwer M, Godard C, Van Eechoutte M. *Pseudomonas aeruginosa* serotype

O12 outbreak studied by arbitrary primer PCR. J Clin Microbiol 1994;32:666–71

97. Kerr JR, Moore JE, Curran MD, Graham R, Webb CH, Lowry KG, et al. Investigation of a nosocomial outbreak of *Pseudomonas aeruginosa* pneumonia in an intensive care unit by random amplification of polymorphic DNA assay. J Hosp Infect 1995;30: 125–31.
98. van Belkum A, Kluytmans J, van Leeuwen W, Bax R, Quint W, Peters E, et al. Multicenter evaluation of arbitrarily primed PCR for typing of *Staphylococcus aureus* strains. J Clin Microbiol 1995;33:1537–47.
99. Reznicek M, Bale M, Pfaller M. Application of DNA probes to antimicrobial susceptibility testing of *Legionella pneumophila*. Diag Microbiol Infect Dis 1991;14:7–10
100. Youmans GR, Davis TE, Fuller DD. Use of chemiluminescent DNA probes in the rapid detection of oxacillin resistance in clinically isolated strains of *Staphylococcus aureus*. Diag Microbiol Infect Dis 1993;16:99–104
101. Ferretti JJ, Gilmore KS, Courvalin P. Nucleotide sequence analysis of the gene specifying the bifunctional 6'-aminoglycoside acetyltransferase 2''-aminoglycoside phosphotransferase enzyme in *Streptococcus faecalis* and identification and cloning of gene regions specifying the two activities. J Bacteriol 1986;167: 631–8.
102. Rood JI, Jefferson S, Bannam TL, Wilkie JM, Mullany P, Wren BW. Hybridization analysis of three chloramphenicol resistance determinants from *Clostridium perfringens* and *Clostridium difficile*. Antimicrob Agents Chemother 1989;33:1569–74.
103. Sanchez-Pescador R, Stempien MS, Urdea MS. Rapid chemiluminescent nucleic acid assays for detection of TEM-1 beta-lactamase-mediated penicillin resistance in *Neisseria gonorrhoeae* and other bacteria. J Clin Microbiol 1988;26:1934–8.
104. Urdea MS, Horn T, Fultz TJ, Anderson M, Running JA, Hamren S, et al. Branched DNA amplification multimers for the sensitive, direct detection of human hepatitis viruses. Nucleic Acids Symp Ser 1991;24:197–200.
105. Arens M. Use of probes and amplification techniques for the diagnosis and prognosis of human immunodeficiency virus (HIV-1) infections. Diag Microbiol Infect Dis 1993;16:165–72.
106. D'Aquila RT. HIV-1 drug resistance. Molecular pathogenesis and laboratory monitoring. Clin Lab Med 1994;14:393–422.
107. Duvoux C, Pawlotsky JM, Cherqui D, Tran van Nhieu J, Metreau JM, Fagniez PL, et al. Serial quantitative determination of hepatitis C virus RNA levels after liver transplantation. A useful test for diagnosis of hepatitis C virus reinfection. Transplantation 1995;60:457–61.
108. Holodniy M, Katzenstein D, Winters M, Montoya J, Shafer R, Kozal M, et al. Measurement of HIV virus load and genotypic resistance by gene amplification in asymptomatic subjects treated with combination therapy. J AIDS 1993;6:366–9.
109. Luque F, Caruz A, Pineda JA, Torres Y, Larder B, Leal M. Provirus load changes in untreated and zidovudine-treated human immunodeficiency virus type 1-infected patients. J Infect Dis 1994; 169:267–73.
110. Eisenstein BI. The polymerase chain reaction: a new method of using molecular genetics for medical diagnosis [Review]. N Engl J Med 1990;322:178–83.
111. White TJ, Madej R, Persing DH. The polymerase chain reaction: clinical applications [Review]. Adv Clin Chem 1992;29:161–96.
112. Loeffelholz MJ, Lewinski CA, Silver SR, Purohit AP, Herman SA, Buonagurio DA, Dragon EA. Detection of *Chlamydia trachomatis* in endocervical specimens by polymerase chain reaction. J Clin Microbiol 1992;30:2847–51.
113. Boddinghaus B, Rogall T, Flohr T, Blocker H, Bottger EC. Detection and identification of mycobacteria by amplification of rRNA. J Clin Microbiol 1990;28:1751–9.
114. Salomon RN. Introduction to quantitative reverse transcription polymerase chain reaction. Diag Mol Pathol 1995;4:82–4
115. Jou NT, Yoshimori RB, Mason GR, Louie JS, Liebling MR. Single-tube, nested, reverse transcriptase PCR for detection of viable *Mycobacterium tuberculosis*. J Clin Microbiol 1997;35: 1161–5.
116. Myers TW, Gelfand DH. Reverse transcription and DNA amplification by a *Thermus thermophilus* DNA polymerase. Biochem 1991;30:7661–6.
117. Young KK, Resnick RM, Meyers TW. Detection of hepatitis C virus by a combined reverse transcription-polymerase chain reaction assay. J Clin Microbiol 1993;31:882–6.
118. Haqqi TM, Sarkar G, David CS, Sommer SS. Specific amplification of a refractory segment of genomic DNA. Nucleic Acids Res 1988;16:11844.
119. Feray C, Sammuel D, Thiers V, Gigou M, Pichon F, Bismuth A, et al. Reinfection of liver graft by hepatitis C after liver transplantation. J Clin Invest 1992;89:1361–5.
120. Roberts TC, Storch GA. Multiple PCR for diagnosis of AIDS-related central nervous system lymphoma and toxoplasmosis. J Clin Microbiol 1997;35:268–9.
121. Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN, Caskey CT. Detection screening of Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res 1988;16: 11141–56.
122. Bej AK, Mahbubani MH, Miller R, Dicesare JL, Haff L, Atlas RM. Multiples PCR amplification and immobilized capture probes for detection of bacterial pathogens and indicators in water. Mol Cell Probes 1990;4:353–65.
123. Geha DJ, Uhl JR, Gustaferro CA, Persing DH. Multiplex PCR for identification of methicillin-resistant staphylococci in the clinical laboratory. J Clin Microbiol 1994;32:1768–72
124. Piatak M, Luk KC, Williams B, Lifson JD. Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species. BioTechniques 1993;14:70–81.
125. Yang L, Weis JH, Eichwald E, Kolbert CP, Persing DH, Weis JJ. Heritable susceptibility to *Borrelia burgdorferi*-induced arthritis is dominant and is associated with persistence of high numbers of spirochetes in tissues. Infect Immun 1994;62:492–500.
126. Coyle MB, Carlson LC, Wallis CK, Leonard RB, Raisys VA, Kilburn JO, et al. Laboratory aspects of "*Mycobacterium genavense*," a proposed species isolated from AIDS patients. J Clin Microbiol 1992;30:3206–12.
127. Relman DA, Loutit JS, Schmidt TM, Falkow S, Tompkins LS. The agent of bacillary angiomatosis: an approach to the identification of uncultured pathogens. N Engl J Med 1990;323:1573–80.
128. Relman DA, Schmidt TM, MacDermott RP, Falkow S. Identification of the uncultured bacillus of Whipple's disease. N Engl J Med 1992;327:293–301.
129. Relman DA, Schmidt TM, Gajadhar A, Sogin M, Cross J, Yoder K, et al. Molecular phylogenetic analysis of the human intestinal pathogen, *Cyclospora*, suggests that it is closely related to *Eimentia*. J Infect Dis 1996;173:440–5.
130. Weyant RS, Hollis DG, Weaver RE, Amin MF, Steigerwalt AG, O'Connor, et al. *Bordetella holmesii* sp. *nov*, a new gram-negative species associated with septicemia. J Clin Microbiol 1995;33:1–7.
131. Tang YW, Hopkins MK, Kolbert CP, Hartley PA, Severance PJ, Persing DH. *Bordetella holmesii*-like organisms associated with septicemia, endocarditis, and respiratory failure [Abstract]. Conference on Molecular Diagnostics and Therapeutics. Washington, DC: American Society of Microbiology, 1997.
132. Van Devanter DR, Warrener P, Bennett LY, Schultz ER, Coulter S,

Garber RL, Rose TM detection and analysis of diverse herpesviral species by consensus primer PCR. J Clin Microbiol 1996, 34:1666–71.

133. Kirschner P, Springer B, Vogel U, Meier A, Wrede A, Kiekenbeck M, et al Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory. J Clin Microbiol 1993;31.2882–9.
134. Neefs JM, Van de Peer Y, De Rijk P, Chapelle S, Wachter R. Compilation of small ribosomal subunit RNA structures. Nucleic Acids Res 1993,21:3025–49.
135. Persing DH, Herwaldt BL, Glaser C, Lane RS, Thomford JW, Mathiesen D, et al. Infection with a babesia-like organism in northern California. N Engl J Med 1995;332:298–303.
136. Monstein HJ, Kihlstrom E, Tiveljung A. Detection and identification of bacteria using in-house broad range 16S rDNA PCR amplification and genus-specific DNA hybridization probes, located within variable regions of 16S rRNA genes. APMIS 1996; 104:451–8.
137. Kwoh DY, Davis GR, Whitefield KM, Chappelle HL, DiMichele LJ, Gingerase TR. Transcription-based amplification systems and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format Proc Natl Acad Sci U S A 1989,86:1173–7.
138. Guatelli JC, Whitfield KM, Kwoh DY, Barringer KJ, Richman DD, Gingeras TR Isothermal in vitro amplification of nucleic acids by multienzyme reaction modeled after retroviral replication Proc Natl Acad Sci U S A 1990,87:1874–8.
139. Compton J. Nucleic acid sequence-based amplification Nature 1991;350:91–2
140. Gingeras TR, Prodanovich P, Latimer T, Guatelli JC, Richman DD, Barringer KJ. Use of self-sustained sequence replication amplification reaction to analyze and detect mutations in zidovudine-resistant human immunodeficiency virus. J Infect Dis 1991,164: 1066–74.
141. Wu DY, Wallace RB. The ligation amplification reaction (LAR)—amplification of specific DNA sequences using sequential rounds of template dependent ligation. Genomics 1989;4:560–9.
142. Schachter J, Stamm WE, Quinn TC, Andrews WW, Burczak JD, Lee HH. Ligase chain reaction to detect *Chlamydia trachomatis* infection of the cervix. J Clin Microbiol 1994;32:2540–3.
143. Barany F. Genetic disease detection and DNA amplification using cloned thermostable ligase Proc Natl Acad Sci U S A 1991;88. 189–93.
144. Chernesky MA, Lee H, Schachter J, Burczak JD, Stamm WE, McCormack WM, Quinn TC. Diagnosis of *Chlamydia trachomatis* urethral infection in symptomatic and asymptomatic men by testing first-void urine in a ligase chain reaction assay. J Infect Dis 1994;170:1308–11.
145. Walker GT, Fraiser ML, Schram JL, Little MC, Nadeau JG, Malinowski DP. Strand displacement amplification—an isothermal, in vitro DNA amplification technique. Nucleic Acids Res 1992;20:1691–6.
146. Walker GT, Little MC, Nadeau JG, Shank DD. Isothermal, in vitro amplification of DNA by a restriction enzyme/DNA polymerase system. Proc Natl Acad Sci U S A 1992;89.392–6.
147. Lizardi PM, Guerra C, Lomeli H, Tussie-Luna I, Kramer FR. Exponential amplification of recombinant-RNA hybridization probes. Biotechnology 1988,6:1197–202.
148. Kramer FR, Lizardi PM. Replicatable RNA reporters. Nature 1989;339.401–2.
149. Prichard CG, Stefano JE. Amplified detection of viral nucleic acid at subattomole levels using Q beta replicase. Ann Biol Chem 1990,48:492–7.
150. Arnold LJ, Hammond PW, Wiese WA, Nelson NC. Assay formats involving acridinium-ester-labeled DNA probes. Clin Chem 1989 35 1588–94.
151. Nelson NC, Kacian DL. Chemiluminescent DNA probes. A comparison of the acridinium ester and dioxetane detection systems and their use in clinical diagnostic assay. Clin Chim Acta 1990,194.73–90.
152. Pollard-Knight D, Read CA, Downes MJ, Howard LA, Leadbetter MR, Pheby SA, et al. Nonradioactive nucleic acid detection by enhanced chemiluminescence using probes directly labeled with horseradish peroxidase. Anal Biochem 1990;34:84–9
153. Mantero G, Zonaro A, Albertini A, Bertolo P, Primi D DNA enzyme immunoassay: general method for detecting products of polymerase chain reaction. Clin Chem 1991;37:422–9
154. Dumler JS, Asanovich KM, Bakken JS, Richter P, Kimsey R, Madigan JE Serologic cross-reactions among *Ehrlichia equi*, *Ehrlichia phagocytophila*, and human granulocytic *Ehrlichia* J Clin Microbiol 1995;33.1098–103
155. Orita M, Hiroyki I, Kanazawa H, Hayashi K, Sekiya T Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci U S A 1989,86.2766–70.
156. Heym G, Honore N, Truffot-Pernot C, Banerjee A, Schurra C, Jacobs WR, et al Implications of multidrug resistance for the future of short-course therapy of tuberculosis: a molecular study Lancet 1994;344:293–8.
157. Felmlee TA, Liu Q, Whelen AC, Williams D, Sommer SS, Persing DH. Genotypic detection of *Mycobacterium tuberculosis* rifampin resistance: comparison of single-strand conformation polymorphism and dideoxy fingerprinting. J Clin Microbiol 1995;33. 1617–23.
158. Van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, et al. Strain identification of *Mycobacterium tuberculosis* by DNA finger-printing: recommendations for a standardized methodology. J Clin Microbiol 1993;31:406–9.
159. Whelen AC, Felmlee TA, Hunt JM, Williams DL, Roberts GD, Stockman L, Persing DH. Direct genotypic detection of *Mycobacterium tuberculosis* rifampin resistance in clinical specimens by using single-tube heminested PCR. J Clin Microbiol 1995;33: 556–61.
160. Jackson KM, Edwards RM, Bowden DS, Leslie DE. Evaluation of the Roche Amplicor polymerase chain reaction system for detection of *Mycobacterium tuberculosis* complex in specimens. Pathology 1996,28 65–7.
161. Ichiyama S, Iinuma Y, Tawada Y, Yamon S, Hasegawa Y, et al. Evaluation of Gen-Probe amplified *Mycobacterium tuberculosis* direct test and Roche PCR-microwell plate hybridization method (Amplicor Mycobacterium) for direct detection of mycobacteria. J Clin Microbiol 1996;34.130–3.
162. Schwab KJ, De Leon R, Sobsey MD. Immunoaffinity concentration and purification of waterborne enteric viruses for detection by reverse transcriptase PCR. Appl Environ Microbiol 1996;62: 2086–94.
163. Sandhu GS, Kline BC, Stockman L, Roberts GD. Molecular probes for diagnosis of fungal infections J Clin Microbiol 1995; 33.2913–9.
164. Fedorko DP, Nelson NA, Cartwright CP. Identification of microsporidia in stool specimens by using PCR and restriction endonucleases. J Clin Microbiol 1995;33.1739–41.
165. Tang YW, Espy MJ, Persing DH, Smith TF. Molecular evidence and clinical significance of herpesvirus infection in the central nervous system. J Clin Microbiol (in press).
166. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 1989;244:359–62.
167. Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, et

al. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 1989;244:362–4.

168. Alter HJ, Purcell RH, Shih JW, Melpolder JC, Houghton M, Choo QL, Kuo G. Detection of antibody to hepatitis C virus in prospec tively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. N Engl J Med 1989;321:1494–500.
169. Aach RD, Stevens CE, Hollinger FB, Mosley JW, Peterson DA, Taylor PE, et al. Hepatitis C virus infection in post-transfusion hepatitis. An analysis with first- and second-generation assays. N Engl J Med 1991;325:1325–9.
170. Nakagiri I, Ichihara K, Ohmoto K, Hirokawa M, Matsuda N Analysis of discordant test results among five second-generation assays for anti-hepatitis C virus antibodies also tested by polymerase chain reaction-RNA assay and other laboratory and clinical tests for hepatitis. J Clin Microbiol 1993;31:2974–80.
171. Hammerle T, Falkner FG, Dorner F. A sensitive PCR assay system for the quantitation of viral genome equivalents: hepatitis C virus (HCV). Arch Virol 1996;141:2103–14.
172. von Herbay A, Ditton HJ, Maiwald M. Diagnostic application of a polymerase chain reaction assay for the Whipple's disease bacterium to intestinal biopsies. Gastroenterology 1996;110: 1735–43.
73. Ramzan NN, Loftus EL, Burgart LJ, Rooney M, Batts KP, Wiesner RH, et al. Diagnosis and monitoring of Whipple disease by polymerase chain reaction. Ann Intern Med 1997;126.520–7.
174. Nocton JJ, Dressler F, Rutledge BJ, Rys PN, Persing DH, Steere AC. Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis. N Engl J Med 1994;330·229–34.
175. Comeau AM, Pitt J, Hillyer GV, Landesman S, Bremer J, Chang BH, et al. Early detection of human immunodeficiency virus on dried blood spot specimens: sensitivity across serial specimens. J Pediatr 1996;129:111–8.
176. Buck GE. Detection of *Bordetella pertussis* by rapid-cycle PCR and colorimetric microwell hybridization. J Clin Microbiol 1996; 34:1355–8.
177. Kellogg JA, Seiple JW, Klinedinst JL, Stroll ES, Cavanaugh SH. Improved PCR detection of *Chlamydia trachomatis* by using an altered method of specimen transport and high-quality endocervical specimens. J Clin Microbiol 1995;33.2765–7.
178. Rota S, Yildiz A, Kustimur S, Akbas E, Gunay A, Guner H. Sample adequacy in detecting *Chlamydia trachomatis*. Int J Gynaecol Obstet 1995;51:225–8.
179. Coccidioidomycosis—United States, 1991–1992. MMWR 1993; 42:21–4.
180. Update. Outbreak of hantavirus infection—Southwestern United States [News]. MMWR 1993,42:477–9.
181. Peerbooms PG, van Doornum GJ, van Deutekom H, Coutinho RA, van Soolingen D. Laboratory-acquired tuberculosis [Letter]. Lancet 1995;345:1311–2.
182. Gruner E, Bernasconi E, Galeazzi RL, Buhl D, Heinzle R, Nadal D. Brucellosis: an occupational hazard for medical laboratory personnel. Report of five cases. Infection 1994;22:33–6.
183. Crotty PL, Staggs RA, Porter PT, Killeen AA, McGlennen RC. Quantitative analysis in molecular diagnostics [Review]. Hum Pathol 1994;25:572–9.
184. Reischl U, Kochanowski B. Quantitative PCR. A survey of the present technology [Review]. Mol Biotechnol 1995;3:55–71.
185. Detmer J, Lagier R, Flynn J, Zayati C, Kolberg J, Collins M, et al. Accurate quantification of HCV RNA from all HCV genotypes using branched DNA (bDNA) technology. J Clin Microbiol 1996; 34:901–7.
186. Pachl C, Todd JA, Kern DG, Sheridan PJ, Fong SJ, Stempien M, et al. Rapid and precise quantification of HIV-1 RNA in plasma using a branched DNA signal amplification assay. J AIDS 1995,8·446–54.
187. Revets H, Marissens D, de Wit S, Lacor P, Clumeck N, Lauwers S, Zissis G Comparative evaluation of NASBA HIV-1 RNA QT, Amplicor-HIV monitor, and Quantiplex HIV RNA assay, three methods for quantification of human immunodeficiency virus type 1 RNA in plasma. J Clin Microbiol 1996,34:1058–64.
188. Saksela K, Stevens CE, Rubinstein P, Taylor PE, Baltimore D. HIV-1 messenger RNA in peripheral blood mononuclear cells as an early marker of risk for progression to AIDS. Ann Intern Med 1995;123 641–8.
189. O'Brien TR, Blattner WA, Waters D, Eyster ME, Hilgartner MW, Cohen AR, et al. Serum HIV-1 RNA levels and time to development of AIDS in the multicenter hemophilia cohort study. JAMA 1996;276:105–10.
190. Mellors JW, Kingsley LA, Rinaldo CR, Todd JA, Hoo BS, Kokka RP, Gupta P. Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion. Ann Intern Med 1995;122:573–9.
191. Mellors JM, Rinaldo CR Jr, Gupta P, White RM, Todd JA, Kingsley LA Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science 1996;272:1167–70.
192. Banerjee SN, Emori TG, Culver DH, Gaynes RP, Jarvis WR, Horan T, et al. Secular trends in nosocomial primary bloodstream infections in the United States, 1980–1989. Am J Med 1991; 91:86S–89S.
193. Pfaller MA, Wenzel RP. Impact of the changing epidemiology of fungal infections in the 1990s. Eur J Clin Microbiol Infect Dis 1992;11:287–9.
194. Troy CJ, Peeling RW, Ellis AG, Hockin JC, Bennett DA, Murphy MR, Spika JS. *Chlamydia pneumoniae* as a new source of infectious outbreaks in nursing homes. JAMA 1997;277: 1214–8.
195. Ahluwalia G, Embree J, McNicol P, Law B, Hammond GW. Comparison of nasopharyngeal aspirate and nasopharyngeal swab specimens for respiratory syncytial virus diagnosis by cell culture, indirect immunofluorescence assay, and enzyme-linked immunosorbent assay. J Clin Microbiol 1987;25:763–7.
196. Schmidt NJ, Gallo D, Devlin V, Woodie JD, Emmons RW. Direct immunofluorescence staining for detection of herpes simplex and varicella-zoster virus antigens in vesicular lesions and certain tissue specimens J Clin Microbiol 1980;12 651–5.
197. Doebbeling BN, Bale MJ, Koontz FP, Helms CM, Wenzel RP, Pfaller MA. Prospective evaluation of the Gen-Probe assay for detection of legionellae in respiratory specimens. Eur J Clin Microbiol Infect Dis 1988;7:748–52.
198. Rodrigues JN, Amaral JL, Leme IL, Pignatari A, Wey S, Hollis R, et al. Molecular epidemiology and antimicrobial susceptibility testing. Testing of quinolone-resistant *Staphylococcus aureus* strains isolated in Brazil. Diag Microbiol Infect Dis 1993;16:9–16.
199. Musser JM, Kapur V, Peters JE, Hendrix CW, Drehner D, Gackstetter GD, et al. Real-time molecular epidemiologic analysis of an outbreak of *Streptococcus pyogenes* invasive disease in US Air Force trainees. Arch Pathol Lab Med 1994;118:128–33.
200. Bauwens JE, Lampe MF, Suchland RJ, Wong K, Stamm WE. Infection with *Chlamydia trachomatis* lymphogranuloma venereum serovar L1 in homosexual men with proctitis: molecular analysis of an unusual case cluster. Clin Infect Dis 1995;20: 576–81.
201. Arikawa J, Lapenotiere HF, Iacono-Connors L, Wang ML, Schmaljohn CS Coding properties of the S and the M genome segments of Sapporo rat virus: comparison to other causative agents of hemorrhagic fever with renal syndrome. Virology 1990;176: 114–25.
202. Tang YW, Ruo SL, Xu X, Sanchez A, Fisher-Hoch SP, McCormick

JB, Xu ZY. Hantavirus strains isolated from rodentia and insectivora in rural China differentiated by polymerase chain reaction assay. Arch Virol 1990;115:37–46.

203. Nichol ST, Spiropoulou CF, Morzunov S, Rollin PE, Ksiazek TG, Feldmann H, et al. Genetic identification of a hantavirus associated with an outbreak of acute respiratory illness. Science 1993;262:914–7.
204. Reid R, Greenberg M, Jenson AB, Husain M, Willett J, Daoud Y, et al. Sexually transmitted papillomaviral infections. I. The anatomic distribution and pathologic grade of neoplastic lesions associated with different viral types. Am J Obstet Gynecol 1987;156:212–22.
205. Fuchs PG, Girardi G, Pfister H. Human papillomavirus DNA in normal, metaplastic, preneoplastic and neoplastic epithelia of the cervix uteri. Int J Cancer 1988;41:41–5.
206. Lorincz AT, Schiffman MH, Jaffurs WJ, Marlow K, Quinn AP, Temple GF. Temporal associations of human papillomavirus infection with cervical cytologic abnormalities. Am J Obstet Gynecol 1990;162:645–51.
207. Morrison EA, Ho GY, Vermund SH, Goldberg GL, Kadish AS, Kelley KF, Burk RD. Human papillomavirus infection and other risk factors for cervical neoplasia: a case-control study. Int J Cancer 1991;49:6–13.
208. Choo QL, Richman KH, Han JH, Berger K, Lee C, Dong C, et al. Genetic organization and diversity of the hepatitis C virus. Proc Natl Acad Sci U S A 1991;88:2451–5.
209. McOmish F, Yap PL, Dow BC, Follett EA, Seed C, Keller AJ, et al. Geographical distribution of hepatitis C virus genotypes in blood donors: an international collaborative survey. J Clin Microbiol 1994;32:884–92.
210. Yoshioka K, Kakumu S, Wakita T, Ishikawa T, Itoh Y, Takayanagi M, et al. Detection of hepatitis C virus by polymerase chain reaction and response to interferon-alpha therapy: relationship to genotypes of hepatitis C virus. Hepatology 1992;16:293–9.
211. Zein NN, Rakela J, Krawitt EL, Reddy KR, Tominaga T, Persing DH. Hepatitis C virus genotypes in the United States: epidemiology, pathogenicity, and response to interferon therapy. Ann Intern Med 1996;125:634–9.
212. Ohno O, Mizokami M, Wu RR, Saleh MG, Ohba K, Orito E, et al. New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. J Clin Microbiol 1997;35:201–7.
213. Ubukata K, Nakagami S, Nitta A, Yamane A, Kawakami S, Sugiura M, Konno M. Rapid detection of the *mecA* gene in methicillin-resistant staphylococci by enzymatic detection of polymerase chain reaction products. J Clin Microbiol 1992;30:1728–33.
214. Zheng X, Kolbert CP, Delmore P, Arruda J, Lewis M, Kolberg J, et al. Direct mecA detection from blood culture bottles by branched DNA (bDNA) signal amplification [Abstract]. 37th Interscience Conference on Antimicrobial Agents and Chemotherapy. Washington, DC: American Society of Microbiology, 1997.
215. Telenti A, Imboden P, Marchesi F, Schmidheini T, Bodmer T. Direct, automated detection of rifampin-resistant *Mycobacterium tuberculosis* by polymerase chain reaction and single-strand conformation polymorphism analysis. Antimicrob Agents Chemother 1993;37:2054–8.
216. Frost SD, McLean AR. Quasispecies dynamics and the emergence of drug resistance during zidovudine therapy of HIV infection. AIDS 1994;8:323–32.
217. Larder BA, Kohli A, Kellam P, Kemp SD, Kronick M, Henfrey RD. Quantitative detection of HIV-1 drug resistance mutations by automated DNA sequencing. Nature 1993;365:671–3.
218. Palu G, Gerna G, Bevilacqua F, Marcello A. A point mutation in the thymidine kinase gene is responsible for acyclovir resistance in herpes simplex virus type 2 sequential isolates. Virus Res 1992;25:133–44.
219. Pease AC, Solas D, Sullivan EJ, Cronin MT, Holmes CP, Fodor SP. Light-generated oligonucleotide arrays for rapid DNA sequence analysis. Proc Natl Acad Sci U S A 1993;91:5022–6.
220. Field AK, Viron KK. "The end of innocence" revisited: resistance of herpesviruses to antiviral drugs. Clin Microbiol Rev 1994;7:1–13.
221. Gibbs WW. New chip off the old block. Can DNA microprobes do for genetics what microprocessors did for computing? [News]. Sci Am 1996;275:42–4.
222. Ross DW. DNA on a chip [Review]. Arch Pathol Lab Med 1996;120:604–5.
223. Cheng J, Shoffner MA, Hvichia GE, Kricka LJ, Wilding P. Chip PCR. II. Investigation of different PCR amplification systems in microfabricated silicon-glass chips. Nucleic Acids Res 1996;24:380–5.
224. Woolley AT, Mathies RA. Ultra-high-speed DNA sequencing using capillary electrophoresis chips [Review]. Anal Chem 1995;67:3676–80.
225. Lamture JB, Beattie KL, Burke BE, Eggers MD, Ehrlich DJ, Fowler R, et al. Direct detection of nucleic acid hybridization on the surface of a charge coupled device. Nucleic Acids Res 1994;22:2121–5.
226. Maurin M, Roux V, Stein A, Ferrier F, Viraben R, Raoult D. Isolation and characterization by immunofluorescence, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Western blot, restriction fragment length polymorphism-PCR, 16S rRNA gene sequencing, and pulsed-field gel electrophoresis of *Rochalimaea quintana* from a patient with bacillary angiomatosis. J Clin Microbiol 1994;32:1166–71.

THE POLYMERASE CHAIN REACTION: CLINICAL APPLICATIONS

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1. Introduction

In the 6 years that have elapsed since the polymerase chain reaction (PCR) technique was published it has had a major impact on medical research (E6, M8, M9, S1). Previous reviews (E3, G1, P2, W7, W9) have focused on its clinical applications in diagnosing viral and genetic diseases, and several books (E4, E5, I1) provide detailed protocols appropriate for the research laboratory.

This article will cover the principle and practice of PCR in the clinical laboratory and applications for diagnosing viral, bacterial, fungal, and parasitic diseases. Other target amplification strategies, such as the ligase amplification system and transcription-based systems derived from PCR, have been reviewed elsewhere (P2). Applications of the method in the diagnosis of cancer (G1, K3) and genetic disease (R1) were reviewed recently. This article contains an outline of the PCR method and its power and limitations, the key issues involved in using it in the clinical reference laboratory, the challenges ahead in producing diagnostic kits, and an overview of its application in specific infectious disease diagnosis.

One objective of this article is to identify those diseases for which PCR offers an advantage over conventional diagnostic methods. For example, it may be the preferred method for detecting pathogens that are difficult, slow, or impossible to culture. Another objective is to address the issues and technical challenges that must be solved before the method will be widely available in the form of FDA-approved standardized kits suitable for the clinical laboratory. Examples of these challenges include procedures for (1) simplifying specimen preparation; (2) eliminating false positives; and (3) colorimetric detection. Questions that we address include whether PCR is too sensitive, whether more than one gene target is necessary, and how positive results can be confirmed if the method is more sensitive than culture. Finally, although we have not cited all of the published articles on clinical applications of PCR, we have provided summary tables for each of the main areas of disease.

2. Principle of PCR

2.1. The Technique

The polymerase chain reaction, developed at the Cetus Corporation in Emeryville, California, employs the enzymatic amplification of DNA *in vitro* (M8, M9, S1). By synthesizing many copies of a selected DNA sequence, PCR is capable of substantially increasing the quantity of this target DNA segment in a sample. This results in a corresponding reduction in the complexity of the nucleic acid sequences in the sample, i.e., the ratio of target to extraneous sequences is vastly increased. Amplification is performed in discrete cycles, and each cycle can, in principle, double the amount of target DNA. The target is therefore exponentially amplified, such that after n cycles there is $(1 + x)^n$ times as much target as was present initially, where x is the mean efficiency of each cycle.

The principle of the method is shown in Fig. 1 (A1). A target DNA sequence to be amplified is chosen first. The nucleotide sequence of the target DNA may be unknown, but sequences of short stretches of DNA on either side of the target must be known. These sequences are used to design two oligonucleotide primers, which

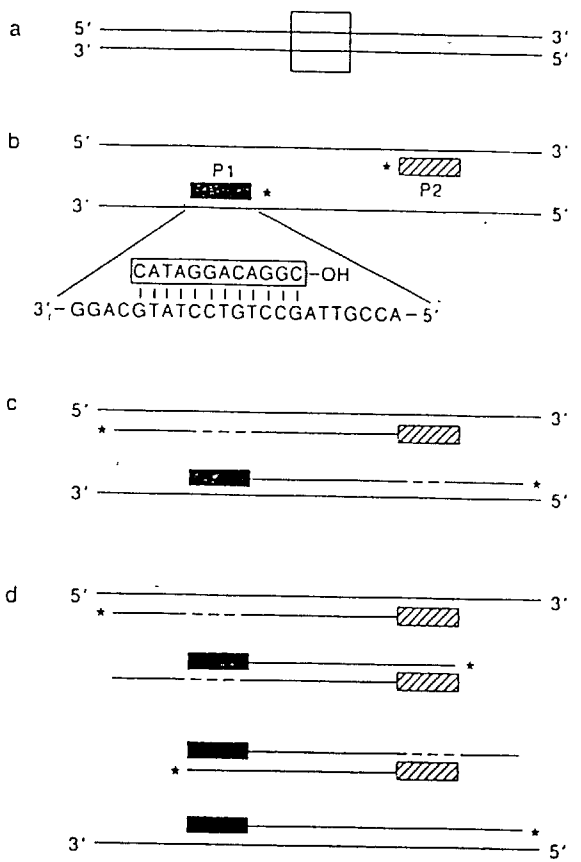

FIG. 1. Principle of the polymerase chain reaction. (a) The target sequence within a double-stranded DNA molecule is indicated with a box. (b) After the DNA has been denatured, the two PCR primers, P1 and P2, anneal to the sequences flanking the target. The 3' end of the primer undergoing elongation by DNA polymerase is denoted by an asterisk. Below primer P1 are the details of the base pairing between the primer (boxed) and the DNA strand. (c) DNA polymerase extends the two primers. The region of the extension product complementary to the other primer is shown by a broken line. (d) In the second cycle of PCR, each of the four DNA strands shown in c anneals to a primer, which is subsequently extended. Note that, at the completion of the second cycle, there are four double-stranded copies of the target, which was originally present as one double-stranded molecule in a. Note also that two of the eight single-stranded products are equal in length to the two primers and the intervening target. Products of this size accumulate exponentially during additional cycles. Reprinted with permission of Arnheim *et al.* (A1) and *BioSciences*.

are single strands of DNA, each approximately 20 nucleotides long. The primers are made by an automated DNA synthesizer. Specific primers can be ordered from commercial suppliers.

Each pair of primers is designed so that the nucleotide sequence of one primer is complementary to sequences flanking one end of the target DNA, whereas the other primer is complementary to the other flanking sequence. After the double-stranded target DNA is denatured into single strands, the primers hybridize to their complementary sequences flanking the target gene. The primers are oriented so that when they bind to the flanking sequences, their 3′-hydroxyl ends face the target sequence. Next, a thermostable DNA polymerase (S2) is added, and, because DNA polymerases extend DNA chains by adding deoxyribonucleoside monophosphates to the 3′ end of each chain, the polymerase extends the primers, thereby making copies of the target. The extension products of each primer must be long enough so that they include the sequences complementary to the other primer.

This series of steps—DNA denaturation, primer hybridization, and DNA polymerase extension—represents a PCR cycle. Each of the three steps must be carried out at an appropriate temperature. Because the products of one cycle can serve as templates for the next, if the first cycle is followed by additional ones, more copies of the target sequence will be made. The main product of the procedure is a double-stranded DNA fragment equal in length to the sum of the lengths of the two primers and the intervening DNA. Single-stranded DNA can also serve as a template, as can RNA after a complementary DNA strand has been synthesized with a reverse transcriptase. Because the quantity of target DNA theoretically doubles with each cycle, as few as 20 cycles generates approximately a million times the amount of target sequence present initially. If only 90% of the targets are extended in each cycle, 20 cycles would yield a 375,000-fold amplification. Nontarget sequences that anneal to one primer and become extended could at most increase 20-fold in concentration during 20 cycles because the product of the first primer extension is not likely to contain the sequence region complementary to the other primer. In some cases, nonspecific binding of the other primer to the extension product of the first primer may result in exponential amplification of nontarget sequences.

Although the theory of PCR is straightforward, a major problem might be expected. In a human genome containing in excess of 10^9 nucleotides, a 1000-base pair target would represent only 10^{-6}–10^{-7} of the available DNA. The annealing of the primer to the many nontarget sequences in such complex templates, even if it occurred infrequently, would lower the purity of the target in the final product. The extent to which imperfect annealing and extension can occur depends on the temperatures during the primer-annealing and polymerase extension steps, because the specificity of primer annealing is greater at higher temperatures. The use of a heat-resistant DNA polymerase allows annealing to

be carried out at an elevated temperature, thus reducing annealing to nontarget sequences. This added selectivity now allows the experimenter to produce large amounts of virtually pure target DNA for characterization. Parameters of the reaction that affect its efficiency include the concentration of enzyme, magnesium ion, and primers. Optimization of the reaction by comprehensive titration of the components is essential for the development of a highly sensitive, reproducible, and robust assay.

2.2. Target Selection

For detecting an infectious disease organism one can choose among several strategies in selecting the genetic target to be amplified. Genes that provide essential functions and contain both conserved (invariant) and variable sequence regions can be targeted. Specificity can be obtained either at the amplification (primer) or detection (probe) stage. Alternatively, the target might be a virulence gene that is uniquely responsible for distinguishing pathogenic from closely related nonpathogenic strains, types, or species. Pathogen-associated targets, such as cryptic plasmid genes, surface membrane protein genes, or randomly cloned sequences, can also be used as long as specificity can be demonstrated. General guidelines for the design of primers have been described (I1).

To illustrate the first strategy for detecting the bacterial pathogen that causes Lyme disease, one could target a segment of the essential, multicopy, small-subunit ribosomal RNA (rRNA) gene and design primers based on sequences that are invariant among all *Borrelia* species, but different from other bacteria outside this genus (P4). The intervening region within the amplified segment can be used to distinguish DNA amplified from *Borrelia burgdorferi* from other *Borrelia* species with a species-specific probe. Variations on this approach can be used at any level of the molecular taxonomic hierarchy, i.e., for bacterial meningitis one could use primers based on rRNA gene sequences that are identical among all eubacteria and identify specific pathogens with probes for *Haemophilus influenza*, *Neisseria meningitidis*, etc. (L1). Alternatively, species specificity can be conferred at the amplification level by using primer–target mismatches to prevent primer extension of sequences from the nontarget organisms (K14, N1, W15). This general approach can be used for viral diseases by targeting regions of essential genes that are either conserved or pathogen specific among, for example, herpesviruses or retroviruses.

Examples of the second approach to target selection are provided by the work of Frankel and co-workers (F2) and Persing *et al* (P3). In the former study, toxin genes and an invasion-associated locus were used to distinguish pathogenic *Escherichia coli* and *Shigella* from normal gut flora. Persing and co-workers (P3) targeted a plasmid-encoded outer-surface protein gene to identify the Lyme disease pathogen. A key factor to consider when selecting sequences for primers and

probes is that most published gene sequences have been obtained from single isolates of a particular bacterial species or virus. Therefore, regardless of the target selected, one must ensure that the primer sequences are conserved in all isolates of the target microorganism by empirically testing many strains and closely related nontarget organisms from different geographic regions.

In developing a PCR assay for a microorganism it is generally advisable to evaluate initially multiple primer pair systems, ideally for different genes, particularly if the primary gene target is nonessential for viability. Multiple primer sets can be used to clarify any negative results that may be due to plasmid target loss, sequence deletion, or unanticipated natural variation. If the data from multiple isolates demonstrate perfect concordance between the primary and confirmatory targets and high clinical sensitivity with a single primer pair/probe system, then the confirmatory sets can subsequently be dropped. Discordance among primer sets may be due to factors other than sequence variability, such as (1) sample bias due to low numbers of target organisms, (2) different analytical sensitivities for different primer sets, and (3) the length of the primer. In this regard, standardized reagents and procedures in the form of approved kits may go a long way toward determining whether different clinical sensitivities reported from various laboratories were due to the absence of a standardized procedure and reagents or to target gene variability. In the largest study reported to date (S9), five laboratories analyzed 200 coded blood samples for HIV-1. One lab had 100% sensitivity and specificity for all samples; in this lab the concordance between two primer pair sets for different regions of the HIV-1 *gag* gene was 100%. The lack of concordance between primer systems in other labs was clearly due to variations in the reagents and procedure rather than to an inherent inability to amplify some isolates with either primer pair. These results, if confirmed with additional HIV specimens, will have important implications for the design, speed, and complexity of PCR tests in the clinical laboratory. Degenerate primers (which are composed of a mixture of oligonucleotides containing a mixture of bases at some positions) offer an additional approach to extending the range of the amplification system to identify isolates with heterogeneous target sequences (C6, K7, M2, N3, S4).

In some cases, intraspecies sequence variability, or "microheterogeneity," may be highly useful for designing probes that can both identify and distinguish pathogenic isolates for epidemiologic studies. For example, PCR confirmed that a patient had become infected with the same strain of HIV-1 that was isolated from her dentist (W11). In another study, Rosa *et al.* (R5) identified sequence variability in a randomly cloned gene from North American and European isolates of *B. burgdorferi*. This variation may prove useful in following the spread of this disease in different geographic areas. Kwok *et al.* (K10, K11) identified variable regions of HTLV that are flanked by conserved regions. Their primers amplify all isolates of HTLV-I and -II while specific probes to the variable sequences are conserved within a type, but can distinguish type I from II.

2.3. Sample Preparation

One advantage that PCR has over many other DNA probe diagnostic methods is that small, degraded, damaged, and unpurified DNA can still serve as a template for the first cycle of amplification. Because subsequent cycles mainly use the newly synthesized product of previous cycles as template, poor-quality targets are irrelevant once amplification is underway.

Simple dilution of crude extracts of clinical specimens often eliminates inhibitors of the amplification reaction while still providing enough initial template for it to proceed. Thus, microorganism DNA or RNA has been amplified directly from crude lysates of human peripheral blood mononuclear cells, bacteria, insect vectors, cervical swabs, urine, hair, sputum, and preserved tissues (W9). Nonetheless, most current procedures for blood-borne viruses such as HIV and HTLV still require a time-consuming isolation of leukocytes prior to the cell lysis step. What is still needed in order to move these relatively cumbersome specimen handling methods out of the skilled reference lab is more rapid, simple, and preferably automated procedures. Because hematin in blood (H6) is the primary inhibitor of the DNA polymerase in the PCR, perhaps a rapid method will be discovered for inactivating it (L2). Then it might be possible to consider detecting a single bacterium (which contains 15,000 copies of rRNA) in 10 ml of blood from a sepsis patient, because a 50-μl aliquot of a completely lysed and homogeneous sample would contain 75 copies of the target.

Another promising approach to sample preparation is capture probe technology. In this method the target DNA is hybridized to a probe attached to a magnetic bead. Nontarget sequences are removed by washing prior to amplification. This procedure was developed in conjunction with the Qβ signal amplification system (G2, H10). Target capture may prove to be a necessary step for concentrating small numbers of target molecules from a biological fluid. For efficient recovery of *Listeria monocytogenes*, 10 ml of cerebrospinal fluid is required. Likewise, increasing the volume of blood culture inoculum from 5 to 10 ml substantially improves bacterial recovery. Such large volumes are cumbersome for DNA extraction; thus a magnetic target capture system following chaotropic lysis may improve assay sensitivity and reduce sampling error. This source of error becomes important when testing small amounts of specimens that may have minute amounts of target (i.e., 1–10 molecules), because variation can occur simply from nonuniform distribution of the target throughout the sample. Thus far, the capture probe approach has not been reported to detect fewer than several thousand copies of target, though this limitation might be a feature of the Qβ system due to the inability to remove nonspecifically bound, but still amplifiable, detection probe. However, in combination with PCR, the capture probe sample preparation technology might be capable of detecting 10 or fewer molecules of target.

One implication of the high sensitivity of PCR is that it may alter patient sampling requirements so that they can be convenient and less invasive. Buccal epithelial cells derived from a mouthwash have been used successfully to identify carrier status for a cystic fibrosis mutation. Hair samples from a patient, relatives at geographically distant locations, and unrelated donors can be rapidly analyzed for histocompatibility leukocyte antigen (HLA) genotype in order to identify compatible donors for bone marrow transplantation (Table 1). Urinary sediments might be used in place of urethal swabs in the diagnosis of infectious urethritis, peripheral blood might be used instead of bone marrow or liver biopsy in the diagnosis of atypical mycobacterial infections, and peripheral blood mononuclear cells (PBMCs) may be used instead of bone marrow in the detection of recurrent leukemias or lymphomas. The ability to HLA type a patient's specimen also offers the potential for resolving instances of sample mix-up, i.e., a particular specimen can be unequivocally associated with a patient of the same type(s).

2.4. Detection of Amplified DNA

The first detection methods used with PCR were radioactively labeled probes to identify specific amplified sequences (M8, S1). With improvements in amplification specificity it became possible to visualize amplified DNA of the predicted size directly by its fluorescence on an agarose or polyacrylamide gel (M9) following staining with ethidium bromide. Probe-based methods remain a key feature of current detection systems primarily because of the additional information and sequence specificity they provide. Probes have been converted to nonisotopic colorimetric systems (B6) by labeling them with an enzyme such as

TABLE 1
HLA Genotyping at the DQA, DPB, and DRB Loci Using DNA Amplified from Single Hairs of Relatives of a Bone Marrow Transplant Candidate

Individual	DQA type	DPB type	DRB type
Patient	4,4	1,1	3,5 (DRB1.0301/1102)
Parent	4,4	4.1,1	NT[b]
Sibling	4,4	2,1.1	5,5 (DRB1 1101/1102)
Sibling[a]	4,4	1,1	3,5 (DRB1·0301/1102)
Son	3,4	4.1,1	NT
Daughter	3,4	4.1,1	NT
Cousin	1.1,4	2 1,14	NT

[a]This sibling inherited the same chromosomes 6 and was a candidate donor for bone marrow transplantation. Subsequent analysis of the class I loci confirmed their genetic identity at the relevant transplantation loci.

[b]NT, Not tested.

horseradish peroxidase. In another approach, the probe is "reversed" or bound to a membrane, where it "captures" a specific allele or sequence variant if it is present in the amplified DNA (S3). This reverse dot-blot format is currently available as a kit for HLA-DQA genotyping and offers the conceptual and practical advantage of simultaneously allowing the detection of multiple alleles (or different pathogens) in a single hybridization reaction (Fig 2). Probes have also been bound to the wells of microtiter plates (H5). This format (Fig 3) has certain advantages for automation with equipment already present in clinical labs such as liquid-transfer devices, plate washers, and readers.

Another probe-based method of detection that looks promising for automation is the colorimetric oligonucleotide ligation assay (OLA), shown in Fig. 4 (N2). This format employs two adjacent oligonucleotides, a 5'-biotinylated probe (with its 3' end at the nucleotide to be assayed) and a 3' reporter probe that is labeled with an enzyme. The probes are hybridized to the amplified target DNA, and if there is perfect complementarity, DNA ligase covalently joins the two probes. Conversely, if there is a mismatch at the junction, ligation is prevented. Capture of the biotinylated, ligated probes on immobilized streptavidin and colorimetric detection of the reporter have been automated.

A luminescent probe detection system called the hybridization protection assay, or HPA, makes use of an acridinium ester-derivatized oligonucleotide that is hybridized to the amplified DNA (O2). Unhybridized probe is preferentially

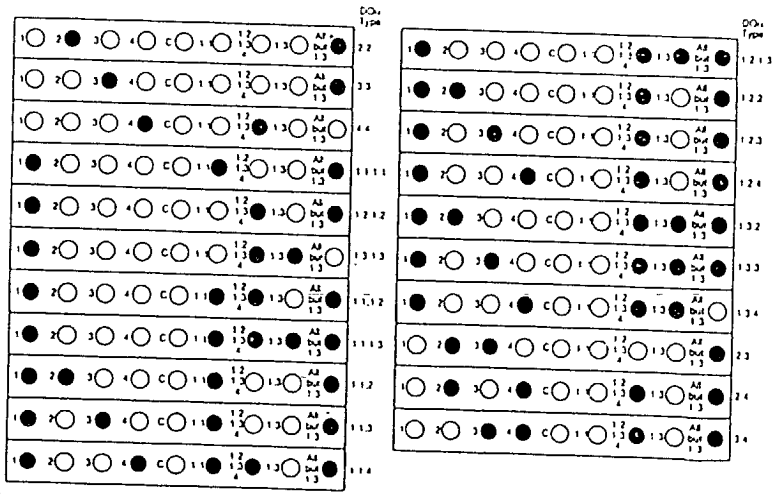

FIG 2 Reverse dot-blot colorimetric detection format for analysis of HLA-DQA genotypes (S3) Reprinted with the permission of Cetus Corporation

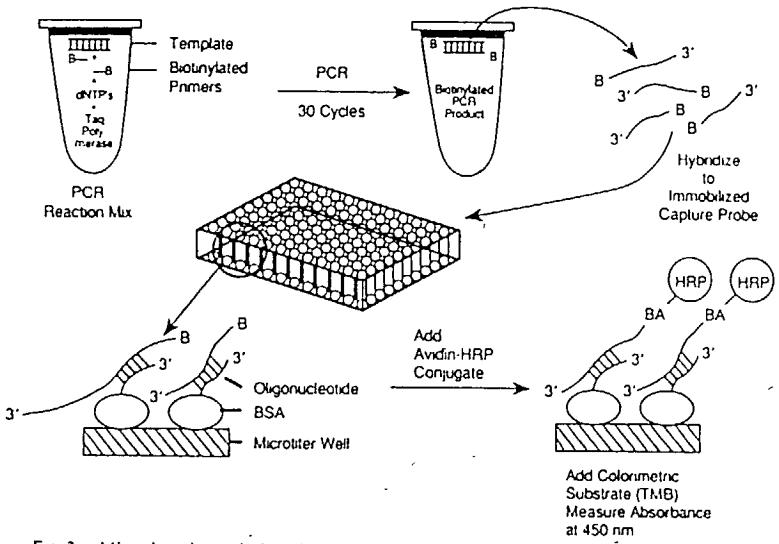

FIG. 3. Microtiter plate colorimetric assay format. Specific probe is bound to the wells of the plate and hybridizes to the amplified, biotinylated DNA target. Unbound primers are removed by washing and the DNA is detected with avidin–horseradish peroxidase and a chromogenic substrate. Reprinted with the permission of Herman *et al.* (H5) and The American Association of Clinical Chemistry.

converted to a form that does not emit light. This format is convenient, rapid, and capable of detecting single nucleotide substitutions, but presently has a limited throughput because each sample must be individually read in a luminometer. An instrument that lends itself to batch processing would enable this detection technology to find wider use in clinical laboratories.

An alternative to probe-based detection systems uses labels on the primers and strives for perfect target specificity in the amplification reaction. This is straightforward if the target gene differs from unintended targets due to the presence of a deletion or gene rearrangement. However, it may require special conditions such as sequence-specific PCR (N1, W15) or "nested" PCR (two sets of primers, one set internal to the first set, in two sequential reactions) (M8) to discriminate between target sequences that differ by only one or a small number of nucleotides. Chehab and Kan (C2) used this approach by derivatizing primers with different fluorescent dyes and scoring the fluorescence relative to the amplified mutant or normal allele in a fluorimeter (Fig. 5). This method has been automated using the Duchenne muscular dystrophy gene deletions as a model. It should prove highly useful in forensic investigations for rapid analysis of amplified targets that differ in length, such as variable-number tandem repeat (VNTR) loci.

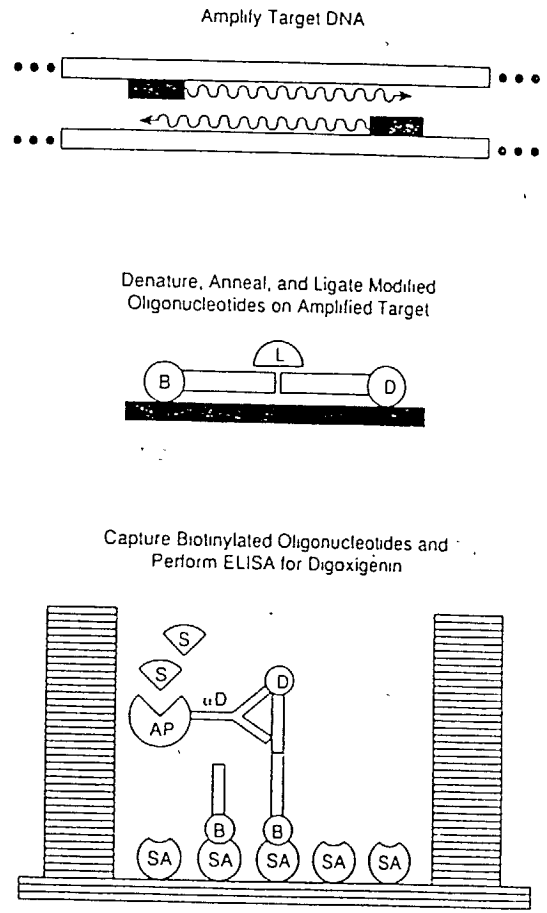

FIG. 4. Schematic diagram of the steps in the automated PCR/OLA procedure performed with a robotic workstation. The assay contains three steps: (1) DNA target amplification, (2) analysis of target nucleotide sequences with biotin (B)-labeled and digoxigenin (D)-labeled oligonucleotide probes and T4 DNA ligase (L), and (3) capture of the biotin-labeled probes on streptavidin (SA)-coated microtiter wells and analysis for covalently linked digoxigenin by using an ELISA procedure with alkaline phosphatase (AP)-conjugated antidigoxigenin (αD) antibodies and a substrate (S). Reprinted with the permission of Nickerson *et al.* (N2) and the *Proc. Natl. Acad. Sci. (U.S.A.)*

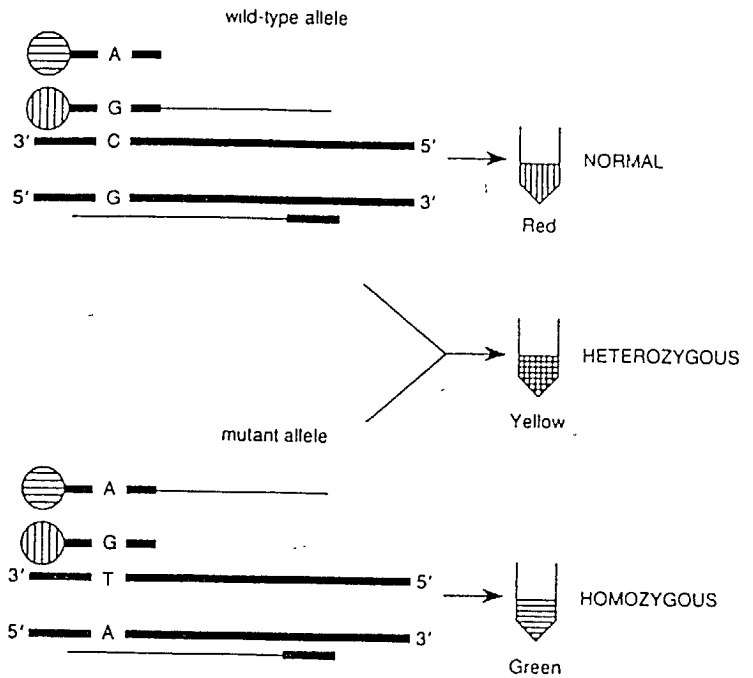

FIG. 5. Strategy for color complementation assay detection of point mutations. A cytidine-to-thymidine mutation is illustrated in the example. Two allele-specific primers corresponding to this region are labeled with red dye (corresponding to the wild-type allele) or green dye (mutant allele). The primer amplifying the opposite strand is unlabeled. After PCR and removal of unincorporated primers, the amplified products for normal, heterozygous, and homozygous DNA are red, yellow, and green, respectively. Reprinted with the permission of Chehab and Kan (C2) and the *Proc. Natl Acad. Sci. (U.S.A.)*.

Kemp and co-workers (K6) developed a colorimetric detection system that incorporates biotin into one (nested) primer and the sequence for a DNA-binding protein (e.g., the *GCN4* gene from *Saccharomyces cerevisiae*) into the other primer. Amplified DNA is captured on an immobilized affinity reagent and the biotinylated product is detected with avidin–horseradish peroxidase and a chromogenic substrate.

2.5. INACTIVATION OF AMPLIFIED DNA

There are three types of sample contamination that can result in false positives with DNA amplification methods. Two types are familiar to clinical chemists. For example, sample-to-sample contamination occurs when a positive specimen contaminates a negative one during sample preparation or during the procedure. Another possible source of sample contamination is from nonviable organisms previously grown or prepared in the sample preparation area. Because some PCR tests are optimized in sensitivity to the level of being able to detect 1–10 molecules of template, it is important with such tests to use positive-displacement pipets when aliquoting clinical specimens or when isolating nucleic acids from them. For less sensitive tests, i.e., genetic disease analyses that may start with several hundred thousand molecules of target (e.g., in a 1 μg of total DNA), such precautions are important but less critical. Pipets that have been used on amplified DNA must never be used for isolation or aliquoting of sample DNA.

The second type of false-positive result can occur at the detection stage, e.g., when the liquid-transfer device pipets a strong positive sample followed by a negative sample. Because a typical PCR reaction can produce 10^{11}–10^{12} molecules of amplified DNA in a 100-μl reaction and a radioactive probe can detect about 10^7 molecules, it is necessary to limit liquid carryover to less than 0.001–0.01 μl (Table 2). Such levels are possible with available automated pipeting devices, but manual pipeting should employ positive-displacement pipets or tips that prevent aerosols from contaminating the device.

TABLE 2
RELATIONSHIP BETWEEN VOLUME OF PCR REACTION CARRYOVER NUMBER OF MOLECULES PRESENT, AND DETECTABILITY BY HYBRIDIZATION TO A RADIOACTIVE PROBE OF MOLECULES CARRIED OVER[a]

Volumes of carryover (μl)	Molecules of carryover	Direct hybridization detection (cpm)
100	10^{12}	11×10^6
10	10^{11}	1.1×10^6
1	10^{10}	1.1×10^5
0.1	10^9	11,000
0.01	10^8	1,100
10^{-3}	10^7	110
10^{-4}	10^6	—
10^{-5}	10^5	—
10^{-6}	10^4	—
10^{-7}	10^3	—
10^{-8}	10^2	—
10^{-9}	10	—
10^{-10}	1	—
10^{-11}	<1	—

[a]Reprinted with the permission of Cimino *et al.* (C4) and *Nucleic Acids Research*

A third type of contamination is unique to PCR and other amplification methods, such as the ligase chain reaction. It involves the inadvertent contamination of a new reaction with the aerosolized products of a previous reaction. As shown in Table 2, as little as 10^{-7} μl of a tube of amplified DNA can contain 10^{1} molecules of target (C4). Recommended precautions (K13) involve the use of positive-displacement pipets and the physical separation of areas where PCR reactions are analyzed from those where new reactions are setup. In laboratories that use these precautions, contamination is infrequent, and, when it does occur, is usually at the 1- to 100-molecule level. However, in addition to these procedural measures, it would be useful to have chemical or enzymatic methods of selectively inactivating amplified DNA—similar to the sterilization procedures used to inactivate large numbers of cultured viruses or bacteria.

Procedurally, there are two points in the assay where inactivation of amplified DNA can be implemented: during setup of a new reaction or at the end of the reaction prior to the detection step. Exploiting principles of the restriction modification and excision repair systems of cells, two groups independently developed a pre-PCR procedure (Fig. 6) that leads to the specific degradation of polynucleotide products from previous reactions but does not affect nucleic acid templates from the clinical specimen (L3, J. Sninsky, unpublished data). Deoxy-UTP (dUTP) is substituted for dTTP and is incorporated into the amplified DNA. In setting up a new reaction, the reagent mixture in the tube contains the enzyme uracil *N*-glycosylase (UNG), which catalyzes the excision of uracil from single- and double-stranded DNA (but not RNA) prior to initiating the temperature cycling process. The resulting abasic polynucleotides are refractory to amplification. This is due to the stalling of the DNA polymerase and/or strand scission because of the alkaline lability of the aglycosidic linkage at the elevated temperature of the first denaturation step. The high denaturation temperature also inactivates the UNG. Conditions have been identified in which every molecule of greater than 10^{6} dUTP-containing templates added to a new reaction can be inactivated. This solution to the carryover of PCR products into reactions about to undergo amplification has several attractive features: (1) both single- and double-stranded DNA contaminants from previous PCR reactions are inactivated, (2) the similarity of the A:U and A:T base pairs in the amplified DNA function in a manner equivalent to hybridization targets, and (3) uracil-containing DNA can be readily cloned and sequenced. Deoxy-UTP incorporation and UNG treatment promise to dramatically reduce false positives in all applications of PCR and thereby lead to its even broader use (J. Sninsky, unpublished data).

Another pre-PCR sterilization process utilizes short-wavelength ultraviolet irradiation of the reaction mixture prior to amplification (S6). Although this procedure can inactivate long DNA fragments, or small numbers of shorter fragments (S7), it is ineffective for more than 10^{3} molecules and requires that both the DNA polymerase and the target nucleic acid be absent from the reaction mixture during

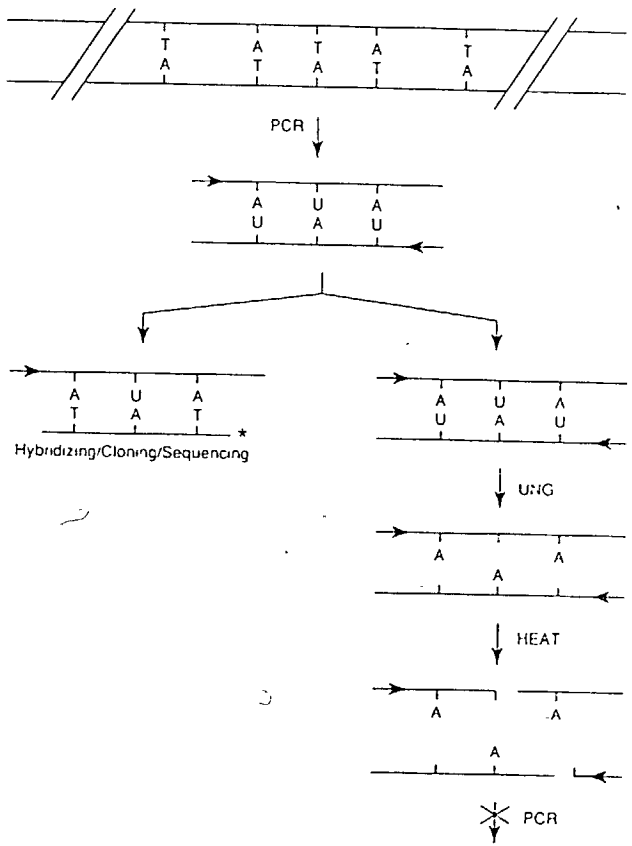

FIG. 6. Schematic representation of the dUTP/uracil-*N*-glycosylase "sterilization" procedure. Reprinted with the permission of Sninsky *et al* (S11)

irradiation (C3). Because the PCR tube must be opened to add these components following irradiation, contamination can still occur.

Cimino and co-workers (C4, I2) developed a post-PCR photochemical procedure for the inactivation of polynucleotides. The procedure is based upon the blockage of *Taq* DNA polymerase when it encounters a photochemically modified base in a polynucleotide strand. Isopsoralen reagents that are added to a reaction mixture prior to amplification tolerate the thermal cycles, are photoactivated after amplification, and form cyclobutane adducts with pyrimidine bases in the DNA.

If the damaged strand is carried over into a new reaction vessel, it is prevented from functioning as a template for amplification. Effective sterilization requires the use of these reagents at concentrations that are tailored to the length and sequence of the target and the level of amplification (Table 3).

2.6. Future Improvements in Technology

In addition to predictable improvements in speed, simplicity, and automation of PCR diagnostic tests, there are several improvements in the procedure that will find use in the reference laboratory prior to their incorporation into kits. First, it has been shown that considerable improvement in target specificity and concomitant improvements in specific product yield and test sensitivity can be achieved by adding or "activating" the thermostable DNA polymerase before the first cycle at a temperature at or above the primer-annealing temperature (F1). This activation can be accomplished in several ways: (1) by adding the enzyme after the reaction has reached an elevated temperature and (2) by sequestering the primers by

TABLE 3
Expected Number of Nonsterilized PCR Molecules as a Function of PCR Product Length[a]

Length of PCR product	Average effective adducts/strand	Nonsterilized molecules per 6×10^{11} molecules
Case A (1 adduct per 5 bases)		
100	10	2.7×10^{7}
150	20	1.2×10^{3}
200	30	<1
250	40	<<1
300	50	<<1
Case B (1 adduct per 15 bases)		
250	13 3	9.7×10^{5}
300	16 6	3.4×10^{4}
350	20	1.2×10^{3}
400	23.3	4.4
450	26.6	1.5

[a]Photochemical sterilization with isopsoralens is a statistical process characterized by measuring the average number a of adducts per strand of amplified DNA assuming a 50% A:T content and a symmetrical distribution of Ts. If the addition reaction is governed by Poisson statistics, the fraction of molecules with no modifications in a large population of amplified DNA molecules that has an averge of a modifications is given by $f_a(0) = e^{-a}$. Reprinted with the permission of Cimino *et al.* (C4) and *Nucleic Acids Research.*

binding them with a single-stranded DNA-binding protein (H9) that is inactivated at high temperature. These approaches avoid any nonspecific priming and synthesis that occur during the reaction setup and during the first rise in temperature to denature the template.

Another improvement for tests when the initial template is RNA rather than DNA is to have a single thermostable enzyme that can function both as a reverse transcriptase and as a DNA polymerase (M12). This would greatly simplify tests for RNA viruses, for bacterial targets that utilize ribosomal RNA targets, and for cancer tests such as for the hybrid mRNA associated with the *BCR–ABL* chromosomal translocation in chronic myeloid leukemia.

Nested PCR reactions can offer significant advantages in sensitivity and specificity yet are prone to carryover contamination of the second reaction that uses the internal primer set. Such contamination cannot be prevented by the sterilization methods described above. Future nested systems may incorporate both the external and internal primers in the same reaction, wherein intentional differences in annealing and denaturation temperatures of the respective primers or target sequences would only permit the intended target to function in primer annealing, denaturation, and synthesis (E6, Y2).

Finally, a major improvement would occur if quantitative procedures (G3, K4, S8, W3) could be simplified and especially if potentially homogeneous methods could be devised (H7). This would greatly extend the utility of diagnostic tests for measuring response to therapy or recurrent disease, and would help address the relationship of pathogen load to clinically significant disease. Like culture, PCR can be optimized to detect single microorganisms and quantitative experiments might offer the opportunity to distinguish septicemia from transient bacteremia (H4) or acute from chronic or latent viral infections. Once the baseline numbers are obtained for a particular microorganism/disease, either (1) the desired level of sensitivity of a qualitative PCR test can be fixed by adjusting parameters such as cycle number or (2) appropriate standards can be run to determine absolute or relative infection levels. Although the potential for detecting nonviable microorganisms following antibiotic therapy is a possible drawback to PCR, a recent study suggests otherwise. Claas and co-workers (C5) found complete agreement between the results of a *Chlamydia* culture technique and PCR in a follow-up study of patients treated with doxycycline. No chlamydial DNA was detected in the patients' samples after treatment. Thus, in our view, the "PCR is too sensitive" critique will become as obsolete as when the same argument was applied to bacterial or viral culture.

A final area for improvement is test turnaround time. Although many current procedures involve 1–2 hours of sample preparation, 2–3 hours of thermocycling, and 1–12 hours for detection, there is nothing sacred about these times. Some whole-blood sample preparation procedures can be done in 1–15 minutes and several nonisotopic detection methods for amplified DNA have been described

that can be completed in 15 minutes. Thermocycling times have to date been limited by the slowness of temperature changes using available instruments, but a recent paper demonstrated that it is possible to reduce each complete cycle of denaturation, annealing, and synthesis to as few as 20 seconds (W12). Because a single molecule of template can be amplified to the point of being visualized by fluorescence on a gel after 42–45 cycles at 85% efficiency, it is possible to consider reducing test thermocycling time to approximately 30 minutes. A specimen-to-result turnaround time of 1 hour is theoretically possible with appropriate instrumentation. It is more likely that times of 2–6 hours will be the practice for several more years.

3. PCR in the Clinical Laboratory: Practical Considerations

Clinical reference laboratories are currently performing PCR test services for infectious diseases, genetic diseases, and oncology. These clinical research formats are being streamlined and developed for FDA approval and subsequent availability as kits to all clinical laboratories. Reference laboratories have become an important element in learning about the reliability of this technology. Though the results of tests in the current research format are not treated as clinically diagnostic, the tests are being performed under the rigors of the clinical laboratory. The complexity of these assays has required the technical and theoretical education of the staff performing them, and the cumulative expertise of these personnel has led to specific recommendations in the areas of quality assurance, sample preparation, amplification, detection, and interpretation of results. The typical protocols currently being used in reference laboratories are reviewed below.

3.1. Introduction

In this section of the protocol, the clinical and epidemiologic significance of the PCR test is described, with special attention to the contribution of PCR results to a possible diagnosis. The theory and methodology of the specific assay are also described, including the primers and probes that are used, whether the system employs simultaneous amplification of several targets, the species or allelic differences that are detected, and the detection system that is employed. An assay flow chart (Fig. 7) can be helpful in orienting the technicians to the relationships among the steps of a complex procedure.

3.2. Quality Assurance of Reagents and Equipment

Reference laboratories performing PCR assays in the research format make, test, and cross-over many of their own reagents. Even reagents that are purchased

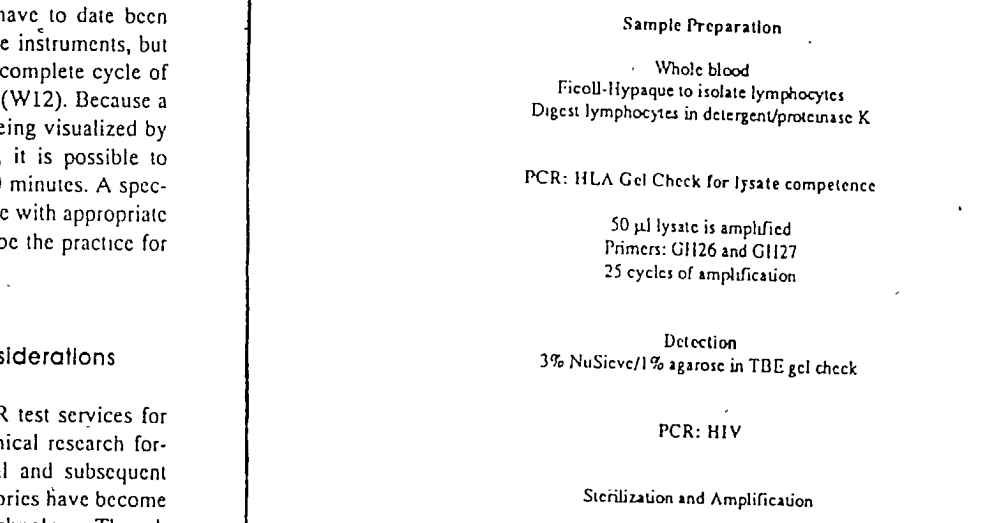

FIG. 7. Assay flow chart.

(e.g., oligonucleotides and enzymes) are tested in-house with recommended quality assurance procedures. Future licensed test kits will not require extensive preparation of reagents, as they will be provided by the manufacturer.

Detailed recommendations pertaining to equipment and supplies should also be given in the protocols. Positive-displacement pipets are crucial for successful PCR operations; temperature-cycling instruments must meet certain minimum stan-

dards. Specifications for detection format equipment (e.g., electrophoresis or transfer apparatus, membranes, probe-labeling supplies) are necessary. Compliance with these requirements contributes to the level of standardization among reference laboratories performing PCR assays.

3.3. Sample Preparation

Sample preparation methods in PCR diagnostics may be different from those that the clinical laboratory has customarily encountered, including the use of unconventional specimens such as hair, chorionic villi, or synovial fluids. In essence, the target DNA or RNA within a clinical specimen must be released and stabilized. The predicted presence of the target nucleic acid defines the sample to be used. In some cases this will entail isolating a group of cells and lysing them, or pelleting samples and treating the pellets, or digesting tissue. An example of a sample preparation method is the one used for the detection of HIV-1 in clinical samples (K12). For this assay, peripheral blood mononuclear cells are isolated from whole blood by density-gradient centrifugation using Ficoll–Hypaque. These cells are then lysed by proteinase K and detergents that release the proviral DNA for amplification.

3.4. Setup and Amplification

Specific instructions must be given to combine the sample DNA with the buffers, dNTPs, specific primers, and DNA polymerase used in the amplification reaction. Each of the components of the reaction has previously been carefully optimized to amplify the intended target efficiently and to prevent extraneous amplification of nonspecific targets. The times and temperatures of each step of an amplification cycle are also optimized specifically for each assay. The information gained during optimization experiments should be described in a robustness table that delineates the tolerance ranges for the particular system. Table 4 is an example generated for a viral pathogen/β-globin coamplification system. In this test, in which the presence of an infectious virus is being examined, the β-globin gene amplification serves as a positive control for sample preparation and for whether the reaction mixture will allow a target to be amplified if it is indeed present. The β-globin gene is present in the cellular material used as the specimen in this assay. A negative viral result without assurance of amplification by a positive β-globin gene result might be a false negative. Controls for successful sample preparation and amplification such as the β-globin and HLA-DQA gene targets are common in infectious disease PCR assays. For genetic and cancer targets, the normal gene, an invariant section of the target gene, or transcribed RNA is a common control (F6).

TABLE 4
Robustness of a Typical Amplification System

Parameter	Optimum[a]	Tolerance range
$[Mg^{2+}]$	4 m*M*	3–8 m*M*; background increases at 8 m*M*
[dNTPs]	200 μ*M* each	200–400 μ*M*; viral gene yield decreases below 200 μ*M*
Taq DNA polymerase	2.5 U/reaction	2–5 U/reaction; background increases with 5 U; β-globin yield decreases with 5 U
Pathogen primers	50 pmol each/reaction	25–100 pmol each/reaction
Cellular control gene primer	5 pmol each/reaction	2.5–10 pmol each/reaction
Denaturing temperature	95°C	92–98°C; viral gene yield decreases above 98°C
Annealing temperature	55°C	50–55°C; viral gene decreases at 60°C
[NaCl]	—	0–30 m*M*; cannot exceed 20 μl 0.9% saline/reaction
PBS	0	Cannot exceed 10 μl PBS/reaction
[EDTA]	—	0–500 μ*M*
SDS	0	0–0.01%; 0–0.03% with 1% Laureth-12

[a]Parameters optimized by simultaneous β-globin/virus amplification.

The setup and amplification section of a protocol also contains specific recommendations for the prevention of carryover of aerosolized DNA into the new reaction. Dedicated hoods or dead-air boxes are recommended in this step of the procedure. All pipets should be of the positive-displacement type. They should be kept in a dedicated setup hood and should never have previously been used to pipet amplified target. No amplified DNA should ever be brought into this area. During the reaction setup, either dUTP and UNG or isopsoralens may be added.

3.5. Detection/DNA Hybridization

There are currently several methods for analysis of the amplified target DNA. For HIV-1, liquid hybridization with radioactively labeled probes is used (K12). Tests for HLA genes and sickle cell anemia utilize the reverse dot-blot format with a nylon membrane (S3). Each clinical research format has a well-characterized detection method defining the optimum probe concentration, the hybridization times and temperatures, as well as the concentrations of indicator reagents. Table 5 describes the optima and tolerances of a nonradioactive dot-blot assay that uses biotinylated probes and detection by a chemiluminescent substrate and a streptavidin–HRP conjugate.

TABLE 5
OPTIMA AND TOLERANCES IN OLIGONUCLEOTIDE PROBING

Probe	Optimum	Tolerances
Hybridization	Probe concentration, 0.5 pmol/ml final	≥ 1–4 pmol/ml results in higher nonspecific background and occasional nonspecific cross-reactivity to high concentration of target.
	Time, 1–2 hours	≤ 1 hour results in lower sensitivity; at ≥ 2 hours there is no appreciable increase in sensitivity or nonspecific background
	Wash time, 10 minutes, twice 2× SSPE/0.1% SDS	SSC can substitute for SSPE
	Wash temperature, 56°C	At 54–56°C there is no loss of specificity or sensitivity; at ≤ 54°C cross-reactivity may occur; at ≥ 57°C loss of specific signal and decreased sensitivity occurs
Streptavidin horseradish peroxidase (SAHRP) binding	SAHRP concentration, 40 ng/ml final	30–40 ng/ml; ≥ 40 ng/ml results in higher nonspecific background; ≤ 30 ng/ml results in concomitant loss of signal and assay sensitivity

3.6. INTERPRETATION

The research formats presently used in clinical reference laboratories employ complex interpretation schemes, though they have been streamlined to be as decisive as is practical. Compatible with traditional interpretation, the first decision to be made is whether the test is valid through examination of the control results. PCR tests may include controls to test for sample preparation, amplification, and the detection of the amplified DNA. Additionally, for each patient specimen, an internal amplification control (e.g., for normal and mutant genes) may be included. Table 6 lists one interpretation scheme for an HIV assay wherein one primer pair and probe are used in the test and the decision is based on replicate testing.

When these research assays have been developed into licensed diagnostic kits, many of the reagent preparation steps, quality assurance provisions, and interpretation schemes may be invisible to the user. However, generating FDA-approved diagnostic kits involves concerns in addition to those we have just examined. Developers must consider the range of skills of clients likely to use the kits for diagnostic purposes and design formats such that all users can run the tests with confidence. Procedures for the licensed clinical kits must be as concise as

TABLE 6
INTERPRETATION SCHEME FOR AN HIV TEST PROCEDURE

Duplicates: A	B	Action taken
0	0	No repeats (no HIV DNA detected)
+	+	No repeats (HIV DNA detected)
0	+	Re-PCR and oligomer hybridization
+	0	Re-PCR and oligomer hybridization

practical while maintaining reproducibility and accuracy. Kits are run thousands of times before tests are marketed and each step of a test is studied for efficiency and effectiveness.

To monitor each test run, developers must give careful consideration to controls. Manufacturers must assure the quality of specimen preparation reagents, enzyme amplification systems, detection systems, oligonucleotide probes, and DNA controls in the test kit. This means that functional enzymes, DNA, substrates, and other chemicals critically optimized to each other must remain so throughout shipping, storage, and use, and the clinical laboratory must be able to evaluate this stability. PCR assay controls are useful for both the amplification and detection systems, because one or both of these components could be at fault if the test does not work. Controls for testing amplification might include one to test the operation of the thermal cycler, one for DNA polymerase function, and a control to test the amplification competence of each specimen. Traditional controls to verify detection system reagents are employed specifically for each type of system (e.g., a color development control for a colorimetric format). A particular challenge to manufacturers is to develop robust kits and to select the appropriate controls to test each component of the system without overwhelming each run with controls.

In addition to assuring the stability and performance of the reagents within a kit, manufacturers must establish the sensitivity, specificity, reproducibility, and accuracy of the entire procedure. Reproducibility and accuracy must be defined for a test system in which logarithmic signal differences can theoretically occur because of differences in amplification efficiency or initial target numbers. The data gathered by the routine use of PCR in reference laboratories will help manufacturers understand the limitations and attributes of each test when confronted with clinical specimens of variable quality. So far, the optimized research formats are performing as predicted and the sensitivity and reproducibility of the methods should be well established when clinical diagnostic kits are available.

DNA testing is relatively new in diagnostics, and amplification technology is as yet untried in a licensed format. Proficiency panels have been made and used in the reference laboratories for the research formats, and researchers have been constructing and sharing their panels for over 2 years. However, these panels, as

well as their subscribers, vary and they do not approach the rigor present in established clinical proficiency testing. PCR diagnostics will not be fully integrated into the clinical laboratory until there is a way to assure the standardization of the results across all laboratories by independent means. Many of the targets described below are unique and approaches to making and supporting proficiency panels may be complex. It would be judicious to plan for the launch of this technology in the clinical setting by providing laboratories with a means to evaluate their proficiency.

4. Specific Applications

4.1. Diagnosis of Viral Infections

The first medical applications of the polymerase chain reactions were for the diagnosis of genetic disease (e.g., sickle cell anemia), because the mutations could be directly studied, and for the detection of the virus known to cause AIDS, because of the advantage of speed compared to culture and of greater sensitivity relative to viral antigen tests (K12, S1). For viruses, PCR also offers the advantages of the ability to detect dormant viruses and noncultivatable viruses such as human papillomaviruses and some enteroviruses, and the direct detection of the pathogen instead of the host's serological response to an infection. The clinical applications of PCR for diagnosis of viral infections have been recently reviewed (W9) and include detection of neonatal infection, early infection, resolution of indeterminate serologies, viral typing, differentiation of indigenous viruses and vaccine strains, and identification of new agents. Table 7 lists some of the viral diseases that can be diagnosed with PCR.

TABLE 7
Representative Viruses and Associated Diseases that Have Been Detected with PCR[a]

Family	Virus	Disease
Herpesviridae	HSV-1, -2	Encephalitis
	CMV	Deafness
Papovaviridae (papillomaviruses)	HPV	Cervical cancer
Flaviviridae	HCV	Hepatitis
	Enteroviruses	Encephalitis
Retroviridae	HIV-1, 2	AIDS
	HTLV-I, II	ATL/TSP

[a]Reprinted with permission from Williams and Kwok (W9) and Marcel Dekker.

4.2. Bacterial Infections

Because culture is the "gold standard" diagnostic method for most bacterial infections, the greatest potential opportunity for PCR to contribute to clinical medicine is in detecting pathogens that are slow, fastidious, or dangerous to grow, or where other DNA probe methods lack sensitivity. Thus, the first reports addressed pathogens such as mycobacteria, *Chlamydia trachomatis, Legionella pneumophila,* and the Lyme disease pathogen *B. burgdorferi* (B5, P5, R4, S11). Recently, many more papers have appeared on mycobacterial PCR tests. These have targeted a diversity of genes—some tests amplify all mycobacteria but detect *Mycobacterium tuberculosis* via a species-specific probe, and some are specific to *M. tuberculosis* at the level of amplification (E2, R3, S10). A similar range of targets has been used for the Lyme pathogen encompassing both universal, plasmid-borne, and randomly cloned chromosomal genes (P3, P4, R5). For this pathogen, much work remains to be done in defining how often the organism can be detected in various clinical specimens at each stage of the disease. Although PCR can detect the organism in synovial fluid, urine, and cerebrospinal fluid from Lyme disease patients with advanced disease, it is not yet clear what the clinical sensitivity would be from a blood or urine specimen in early disease or in patients with ambiguous neurological symptoms and serology.

Table 8 summarizes PCR applications in detecting bacterial pathogens. Most of these studies concern simple detection of the organism rather than diagnosis and await standardized procedures, simplified sample preparation methods, and colorimetric detection formats before they will be practical for clinical laboratories.

Future opportunities include rapid detection and identification of bacterial causes of sepsis and meningitis, with the ability in the latter to differentiate viral and fungal etiologies. Universal primers have been described that are conserved in all eubacteria tested and a similar situation exists for fungi and enteroviruses (B3, R6, W8). A coamplification test that combines primers for the three types of agents could prove medically useful in selecting therapy if the result can be obtained in 2 hours.

Another opportunity for rapid PCR diagnosis involves antibiotic susceptibility testing (C7). Ounissi and co-workers (O3) have demonstrated concordance between the presence of various genes for antibiotic-modifying enzymes and bacterial sensitivity *in vitro*. By judicious selection of conserved or enzyme-specific gene sequences as primer sites, it is possible to detect, for example, all β-lactamases or only those associated with penicillanase-producing *Neisseria gonorrhoea* (M1, S5). Such tests would have to be used on specimens from fluids that are usually sterile (blood or CSF). Preferably they should be directed toward those pathogens for which the spectrum of resistance mechanisms is restricted and the importance of predicting drug resistance is great, e.g., methicillin-resistant *Staphylococcus aureus* infections (H1, M10), or toward the DNA gyrase A mutation that confers resistance to quinolones.

TABLE 8
DEMONSTRATED AND POTENTIAL CLINICAL UTILITY OF BACTERIAL DIAGNOSIS BY PCR

Pathogen	Associated disease	Clinical utility	References
Mycobacterium tuberculosis	Tuberculosis	More rapid than culture, possibly more sensitive? less hazardous in lab	B5, H2, S10
Mycobacterium leprae	Leprosy	More rapid than culture	H3
Mycobacterium avium	Atypical mycobacterial infections, AIDS	More convenient specimens? definitive species identification	F5
Chlamydia trachomatis	Venereal disease	More rapid than culture, improved sensitivity	B2, C5, D1, D3, F5, G4, O1, W6, W13
Borrelia burgdorferi	Lyme disease	Easier, faster than culture, higher specificity than serology	M3, P3, P4, R4, R5
Mycoplasma pneumoniae	Atypical pneumonia	More sensitive than culture	B2
Mycoplasma genitalium	Urethritis	Study role in disease	J1
Enterotoxigenic *Escherichia coli, vibrio* and *Shigella*	Acute diarrhea	Detect multiple pathogens	F4, K8, O1, V1
Salmonella typhi	Typhoid fever	Monitor carriers	F3
Bordetella pertussis	Whooping cough	Diagnosis	H8
Escherichia coli Shiga-toxin		More rapid than conventional methods	K1
Legionella pneumophila	Pneumonia	More rapid than culture	S11
Rickettsia rickettsii	Rocky Mountain spotted fever	Early detection	C1, T2, W10
Rickettsia conorii	Boutonneuse fever	Choice of therapy	T2
Rickettsia tsutsugamushi	Scrub typhus	Rapid, differential diagnosis	K5
Rickettsia typhi	Murine typhus	Prevalence; vector control	W5
Clostridium difficile	Diarrhea	Prophylactic therapy? distinguish toxigenic strains	K2, W14
Treponema pallidum	Syphilis	Improved sensitivity	B3

With further simplifications of the technology it may be feasible to consider patient-specific microorganism diagnoses, in the sense that the immunoglobulin V–D–J rearrangement that is unique to a B cell leukemia patient can be characterized with respect to its sequence (Y1). This information could be used to monitor response to therapy and to detect residual or recurrent disease. Using similar molecular epidemiological information might someday prove useful in selecting therapy or predicting responsiveness, particularly for those pathogens for which different phylogenetic lines are associated with distinctive clinical symptoms (M11).

4.3. Fungal and Parasitic Infections

The advantages of speed, simplicity, and laboratory safety that PCR offers compared to culture of some bacterial pathogens extend to the diagnosis of many fungal and parasitic infections as well (D2). The rapid and definitive diagnosis of congenital *Toxoplasma gondii* infection from amniotic fluid samples may reduce unnecessary abortions and promote earlier treatment of infected fetuses (B7). An important use is to distinguish pathogenic and nonpathogenic isolates of parasites such as amoeba (M4, T1). Rapid detection and differentiation of the mucocutaneous and sylvatic forms of *Leishmania brasiliensis* permit rapid treatment of the former prior to extensive tissue destruction (L4). Lopez *et al.* (L4) also demonstrated that PCR can be performed in the field and can provide highly useful medical information on diseases common in less developed countries.

PCR is just beginning to have an impact on identification and diagnosis of fungal pathogens such as *Cryptococcus neoformans* (V2) and *Pneumocystis carinii* (W1, W2). Universal fungal primers (M5, W8) and pathogen-specific probes (B4) promise to allow rapid and sensitive diagnosis of fungal sepsis and pulmonary disease. Table 9 summarizes the published applications for diagnosis of fungal and parasitic infections.

5. Conclusion

Several predictions can be made regarding the impact of PCR on the clinical laboratory. First, the diagnostic repertoire of the clinical laboratory will expand, primarily because the applications of PCR in human genetics, cancer, and infectious disease are increasing rapidly. In the clinical microbiology laboratory, it will facilitate the detection of pathogens whose identification has previously been limited by the lack of a practical culture system. This will add to the list of organisms that can be detected, increasing both the services and responsibility of the clinical laboratory. In addition, our list of previously unrecognized or unidentified pathogens will grow (R2). Second, the technological nature of these methods will create a demand for laboratory professionals with training in this technology. Currently, very few pathology residency programs, fellowship training, or medical technology programs offer formal instruction in molecular techniques. Given the potential for widespread applications of these methods in the clinical laboratory, consideration must now be given to the adequate training of these future laboratory directors and staff, knowing that those entering programs now will be directly confronted with this technology when they complete their training. Continuing-education programs will have to be developed that are tailored to the needs of this group of professionals to provide them with an understanding of both the power and limitations of these methods. Third, there will be a need to carefully evaluate the data derived from these new methods in light of

TABLE 9
DEMONSTRATED AND POTENTIAL CLINICAL UTILITY OF FUNGAL AND PARASITIC DIAGNOSIS BY PCR

Pathogen	Associated disease	Clinical utility	References
1. *Cryptococcus neoformans*	Meningitis	Faster than culture, more sensitive than antigen test	V2, M5
2. *Histoplasma capsulatum*	Pulmonary disease	Faster, safer than culture	B4
Blastomyces dermatitidis		Faster, safer than culture	
3. *Coccidioides immitis*			B4
4. *Pneumocystis carinii*	AIDS	More sensitive and specific	
5. *Toxoplasma gondii*	Toxoplasmosis	Fetal infection; more sensitive than fetal IgM, faster than tissue culture	B7, G5
6. *Trypanosoma brucei*	Sleeping sickness	Rapid and sensitive diagnosis	M6
Trypanosoma congolense			
7. *Trypanosoma cruzi*	Chagas disease	Sensitivity; replaces xenodiagnosis	E1, M7
8. *Plasmodium falciparum*	Malaria	Higher sensitivity; detection of pyrimethamine resistance	W4, Z1
9. *Leishmania braziliensis*	Leishmaniasis	Differentiation of cutaneous and sylvatic types	L4
10. *Naegleria fowleri*	Amebic meningoencephalitis	Early diagnosis from CSF?	M4
11. *Entamoeba histolytica*	Hemorrhagic colitis	Differentiation of pathogenic strains	T1

the clinical picture, especially if the performance of PCR-based tests significantly exceeds the "gold standards" currently in place. One can envision that PCR could indeed prove more sensitive than standard methods for the diagnosis of infectious disease, because it is able to detect nonviable organisms. Cooperation of clinicians, researchers, developers, and laboratory professionals will be required to integrate PCR results with the clinical presentation, patient history, supporting laboratory data, and treatment records. Only then will the true clinical significance of PCR results be known. Finally, the introduction of molecular diagnostic techniques will create a concurrent demand for proficiency testing and laboratory site visits administered through independent agencies such as the College of American Pathologists (CAP). Though many tests may be offered on an experimental basis, it would be prudent to begin developing CAP proficiency panels in order to achieve rigorous laboratory standards for both experimental and FDA-approved tests.

Nucleic acid amplification techniques will undoubtedly have a substantial future impact on the practice of laboratory medicine. Ultimately, the spread and acceptance of these techniques will be limited by cost and other considerations.

Although amplification methods are now the standard for most genetic disease tests and are becoming important for many infectious agents, conventional culture for most bacterial pathogens is often rapid, inexpensive, as sensitive, and allows detection of multiple organisms from a single procedure. Culture also allows determination of phenotypic characteristics such as antibiotic resistance, virulence factors, and strain differences, which may be difficult or impossible to determine by amplification alone. For infectious diseases, modern serological techniques, especially latex agglutination and solid-phase antibody methods, are also rapid and less labor intensive. The decision to use an amplification method is thus likely to be dictated by the sensitivity and specificity of the PCR procedure versus the low-cost, time-proven conventional method, factoring in the turnaround time and the clinical need for definitive results. With the evolution of this technology, however, will come increased sophistication and automation of many of the steps involved in PCR technology, resulting in lower per-test costs. This will likely result in increased use and may cause us to consider applying it to new areas. Inevitably, the acceptance and competent application of this technology will lead to great improvements in our diagnostic capabilities and to better clinical understanding.

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REFERENCES

A1. Arnheim, N., White, T., and Rainey, W. E., Application of PCR: Organismal and population biology. *BioScience* 40, 174–182 (1990).
B1. Bernet, C., Garret, M., de Barbeyrac, B., Bebear, C., and Bonnet; J. Detection of *Mycoplasma pneumoniae* by using the polymerase chain reaction *J. Clin. Microbiol.* 27, 2492–2496 (1989).
B2 Bobo, L., Coutlee, F., Yolken, R. H., Quinn, T., and Viscidi, R. P., Diagnosis of *Chlamydia trachomatis* cervical infection by detection of amplified DNA with an enzyme immunoassay. *J. Clin. Microbiol.* 28, 1968–1973 (1990)
B3. Böttger, E. C., Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymically amplified DNA. *FEMS Microbiol Lett.* 65, 171–176 (1989).
B4. Bowman, B., Taylor, J. W., and White, T. J., Molecular evolution of the fungi: Human pathogens *Mol Biol. Evol* 9 (in press)
B5. Brisson-Nöel, A., Lecossier, D., Nassif, X., Gicquel, B., Lévy-Frébault, V., and Hance, A. J., Rapid diagnosis of tuberculosis by amplification of mycobacteria DNA in clinical samples. *Lancet* 2, 1069–1071 (1989)
B6. Bugawan, D., Saiki, R. K., Levenson, C. H., Watson, R. M., and Erlich, H. A., The use of non-radioactive oligonucleotide probes to analyze enzymatically amplified DNA for prenatal diagnosis and forensic HLA typing *Bio/Technology* 6, 943–947 (1988)

B7. Burg, J L., Grover, C. M , Pouletty, P , and Boothroyd, J. C , Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by the polymerase chain reaction. *J Clin. Microbiol.* 27, 1787–1792 (1989).
B8. Bustain, J. M , Grimprel, E., Lukehart, S. A , Norgard, M. V., and Radolf, J. D., Sensitive detection of *Treponema pallidum* by using the polymerase chain reaction. *J. Clin. Microbiol* 29, 62–69 (1991).
C1. Carl, M., Tibbs, C. W., Dobson, M. E., Paparello, S., and Dasch, G. A., Diagnosis of acute typhus infection using the polymerase chain reaction. *J. Infect. Dis.* 161, 791–793 (1990).
C2 Chehab, F. F., and Kan Y. W , Detection of specific DNA sequences by fluorescence amplification: A color complementation assay. *Proc. Natl. Acad. Sci. U.S.A.* 86, 9178–9182 (1989)
C3. Cimino, G. D., Isaacs, S. T., Metchette, K. C , and Zhu, Y. S , More false-positive problems *Nature (London)* 345, 773–774 (1990)
C4. Cimino, G. D , Metchette, K. C., Tessman, J. W., Hearst, J. E., and Isaacs, S. T , Post-PCR sterilization: A method to control carryover contamination by the polymerase chain reaction. *Mucleic Acids Res.* 19, 99–107 (1991).
C5. Claas, H. C. J., Wagenvoort, J. H. T., Niesters, H. G. M., Tio, T. T., Van Rijsoort-Vos, J. H., and Quint, W. G. V., Diagnostic value of the polymerase chain reaction for *Chlumydia* detection as determined in a follow-up study *J. Clin. Microbiol.* 29, 42–45 (1991).
C6. Compton, T., Degenerate primers for DNA amplification. *In* "PCR Protocols: A Guide to Methods and Applications" (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J White, eds.), pp. 39–45. Academic Press, San Diego, California, 1990.
C7. Courvalin, P , Genotypic approach of bacterial resistance to antibiotics. *Antimicrob. Agents Chemother.* 35, 1019–1023 (1991).
D1. Dean, D., Pant, C. R , and O'Hanley, P., Improved sensitivity of a modified polymerase chain reaction amplified DNA probe in comparison with serial tissue culture passage for detection of *Chlamydia trachomatis* in conjunctival specimens from Nepal. *Diagn. Microbiol. Infect Dis* 12, 133–137 (1989).
D2. de Bruijn, M. H L., Diagnostic DNA amplification: No respite for the elusive parasite *Parasitol. Today* 4, 293–295 (1988).
D3. Dutilh, B., Bebear, C., Rodriguez, P., Vekris, A., Bonnet, J., and Garret, M , Specific amplification of a DNA sequence common to all *Chlamydia trachomatis* serovars using the polymerase chain reaction. *Res. Microbiol.* 140, 7–16 (1989).
E1. Eakin, A. E , Bouvier, J., Sakanari, J. A. Craik, C. S., and McKerrow, J. H , Amplification and sequencing of genomic DNA fragments encoding cysteine proteases from protozoan parasites *Mol. Biochem Parasitol.* 43, 1–8 (1990)
E2. Eisenach, K. D., Cave, M. D , Bates, J. H , and Crawford, J T , Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis. J. Infect. Dis.* 161, 977–981 (1990)
E3. Eisenstein, B. I , The polymerase chain reaction. A new method of using molecular genetics for medical diagnosis. *N. Engl. J. Med* 322, 178–183 (1990).
E4. Erlich, H A., Gibbs, R., and Kazazian, H. H , Jr , eds., "Current Communications in Molecular Biology: Polymerase Chain Reaction." Cold Spring Harbor Lab., Cold Spring Harbor, New York, 1988.
E5. Erlich, H. A., ed , "PCR Technology: Principles and Applications for DNA Amplification." Stockton Press, New York, 1989.
E6. Erlich, H. A , Gelfand, D., and Sninsky, J J., Recent advances in the polymerase chain reaction. *Science* 252, 1643–1651 (1991).
F1. Faloona, F , Weiss, S., Ferre, F , and Mullis, K., Direct detection of HIV sequences in blood: High-gain polymerase chain reaction. *Abstr. Int. Conf. Aids, 6th,* San Francisco, June 20–24 (1990).

F2 Frankel, G , Newton, S M C , Schoolnik, G K., and Stocker, B A D , Intragenic recombination in a flagellin gene Characterization of the *H1-j* gene of *Salmonella typhi EMBO J* 8, 3149–3152 (1989)
F3. Frankel, G , Giron, J. A , Valmossoi, J , and Schoolnik, G K., Multi-gene amplification: Simultaneous detection of three virulence genes in diarrhoeal stool. *Mol Microbiol* 3, 1729–1734 (1989)
F4. Frankel, G , Riley, L., Giron, J A., Valmassoi, J., Friedmann, A , Strokbine, N , Falkow, S , and Schoolnik, G K., Detection of *Shigella* in feces using DNA amplification *J Infect Dis* 161, 1252–1256 (1990)
F5 Fries, J W , Patel, R. J , Piessens, W. F , and Wirth, D. F , Genus- and species-specific DNA probes to identify mycobacteria using the polymerase chain reaction *Mol Cell Probes* 4, 87–105 (1990)
F6. Frye, R. A , Benz, C. C., and Liu, E., Detection of amplified oncogenes by differential polymerase chain reaction. *Oncogene* 4, 1153–1157 (1989).
G1. Gibbs, R. A., DNA amplification by the polymerase chain reaction *Anal Chem* 62, 1202–1214 (1990)
G2 Gillespie, D , Thompson, J , and Solomon, R., Probes for quantitating subpicogram amounts of HIV-1 RNA by molecular hybridization. *Mol Cell Probes* 3, 73–86 (1989).
G3 Gilliland, G., Perrin, S., and Bunn, H. F., Competitive PCR for quantitation of mRNA *In* "PCR Protocols: A Guide to Methods and Applications" (M A. Innis, D. H. Gelfand, J J Sninsky, and T. J White, eds), pp. 60–69. Academic Press, San Diego, California, 1990
G4. Griffais, R., and Thibon, M , Detection of *Chlamydia trachomatis* by the polymerase chain reaction *Res. Microbiol* 140, 139–141 (1989)
G5. Grover, C. M., Thulliez, P , Remington, J. S , and Boothroyd, J. C , Rapid prenatal diagnosis of congenital *Toxoplasma* infection by using polymerase chain reaction and amniotic fluid *J. Clin. Microbiol* 28, 2297–2301 (1990).
H1. Hackbarth, C. J., and Chambers, H. F., Methicillin-resistant staphylococci Genetics and mechanisms of resistance. *Antimicrob. Agents Chemother.* 33, 991–994 (1989)
H2. Hance, A. J , Grandchamp, B., Lévy-Frébault, V., Lecossier, D., Rauzier, J , Bocart, D , and Gicquel, B , Detection and identification of mycobacteria by amplification of mycobacterial DNA. *Mol Microbiol* 3, 843–849 (1989)
H3. Hartskeerl, R. A , DeWit, M Y L., and Klatser, P. R., Polymerase chain reaction for the detection of *Mycobacterium leprae J. Gen Microbiol.* 135, 2357–2364 (1989).
H4. Heimdahl, A , Hall, G , Hedberg, M , Sandberg, H , Söder, P., Tunér, K., and Nord, C E., Detection and quantitation by lysis-filtration of bacteremia after different oral surgical procedures *J. Clin. Microbiol* 28, 2205–2209 (1990)
H5. Herman, S , Loeffelholz, M , Silver, S., Purohit, A , Lewinski, C , Buonagurio, D , Molina, M , Rosen, L., Lin, P , Liu, P , Barone, A. D , Keys, L. D., and Longiaru, M , Detection of *Chlamydia trachomatis* in cervical specimens using PCR combined with a rapid sample preparation method and an enhanced microtiter plate detection system *Abstr Conf Nucleic Acids, 5th,* San Diego, Nov. 14–16 (1990)
H6. Higuchi, R., Simple and rapid preparation of samples for PCR *In* "PCR Technology Principles and Applications for DNA Amplification" (H A Erlich, ed.), pp 31–38 Stockton Press, New York, 1989
H7. Holland, P. M , Abramson, R D , Watson, R., and Gelfand, D H , Detection of specific polymerase chain reaction product by utilizing the 5′ → 3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc Natl Acad. Sci. U.S.A* 88, 7276–7280 (1991)
H8 Houard, S , Hacket, C , Herzog, A , and Bollen, A., Specific identification of *Bordetella pertussis* by the polymerase chain reaction. *Res. Microbiol* 140, 477–487 (1989)
H9. Hung, T., Mak, K., and Fong, K., A specificity enhancer for polymerase chain reaction *Nucleic Acids Res* 18, 4953 (1990)

H10. Hunsaker, W. R., Badri, H., Lombardo, M., and Collins, M. L., Nucleic acid hybridization assays employing dA-tailed capture probes *Anal. Biochem.* **181**, 360–370 (1989).

I1. Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds., "PCR Protocols: A Guide to Methods and Applications." Academic Press, San Diego, California, 1990.

I2. Isaacs, S. T., Tessman, J. W., Metchette, K. C., Hearst, J. E., and Cimino, G. D., Post-PCR sterilization: Development an application to and HIV-1 diagnostic assay *Nucleic Acids Res.* **19**, 109–116 (1991).

J1. Jensen, J. S., Uldum, S. A., Sondergåard-Andersen, J., Vuust, J., and Lind, K., Polymerase chain reaction for detection of *Mycoplasma genitalium* in clinical samples. *J. Clin. Microbiol* **29**, 46–50 (1991).

K1 Karch, H., and Meyer, T., Single primer pair for amplifying segments of distinct Shiga-like toxin genes by polymerase chain reaction. *J. Clin. Microbiol.* **27**, 2751–2757 (1989).

K2. Kato, N., Ou, C.-Y., Kato, H., Bartley, S. L., Brown, V. K., Dowell, V. R., Jr., and Ueno, K., Identification of Toxigenic *Clostridium difficile* by the polymerase chain reaction *J. Clin Microbiol.* **29**, 33–37 (1991).

K3. Kawasaki, E. S., The polymerase chain reaction: Its use in the molecular characterization and diagnosis of cancers. *Cancer Invest.* (in press).

K4. Kellogg, D. E., Sninsky, J. J., and Kwok, S., Quantitation of HIV-1 proviral DNA relative to cellular DNA by the polymerase chain reaction. *Anal. Biochem.* **189**, 202–208 (1990).

K5. Kelly, D. J., Marana, D. P., Stover, C. K., Oaks, E. V., and Carl, M., Detection of *Rickettsia tsutsugamushi* by gene amplification using polymerase chain reaction techniques *Ann. N. Y. Acad. Sci.* **590**, 564–571 (1990).

K6 Kemp, D. J., Smith, D. B., Foote, S. J., Samaras, N., and Peterson, M. G., Colorimetric detection of specific DNA segments amplified by polymerase chain reactions. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2423–2427 (1989).

K7. Knoth, K., Roberds, S., Poteet, C., and Tamkun, M., Highly degenerate, inosine-containing primers specifically amplify rare cDNA using the polymerase chain reaction *Nucleic Acids Res* **16**, 10932 (1988).

K8. Kobayashi, K., Seto, K., Ueguchi, M., Makino, M., Ishibashi, M., Akasaka, S., and Yamamoto, K., A new simple method of detecting cholera enterotoxin gene from *Vibrio cholerae* O1 isolates. *Igaku no Ayumi* **150**, 509–510 (1989).

K9 Krawczak, M., Reiss, J., Schmidtke, J., and Rosler, U., Polymerase chain reaction: Replication errors and reliability of gene diagnosis. *Nucleic Acids Res.* **77**, 2197–2201 (1989)

K10 Kwok, S., Ehrlich, G., Poiesz, B., Kalish, R., and Sninsky, J. J., Enzymatic amplification of HTLV-I viral sequences from peripheral blood mononuclear cells and infected tissues. *Blood* **72**, 1117–1123 (1988)

K11. Kwok, S., Kellogg, D., Ehrlich, G., Poiesz, B., Bhagavati, S., and Sninsky, J. J., Characterization of a sequence of human T cell leukemia virus type I from a patient with chronic progressive myelopathy. *J. Infect. Dis.* **158**, 1193–1197 (1988).

K12. Kwok, S., Mack, D. H., Mullis, K. B., Poiesz, B., Ehrlich, G., Blair, D., Friedman-Kien, A., and Sninsky, J. J., Identification of human immunodeficiency virus sequences by using *in vitro* enzymatic amplification and oligomer cleavage detection. *J. Virol* **61**, 1690–1694 (1987).

K13. Kwok, S., Procedures to minimize PCR-product carryover. *In* "PCR Protocols A Guide to Methods and Applications" (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J White, eds.), pp. 142–145. Academic Press, San Diego, California, 1990.

K14. Kwok, S., Kellogg, D. E., McKinney, N., Spasic, D., Goda, L., Levenson, C., and Sninsky, J. J., Effects of primer-template mismatches on the polymerase chain reaction Human immunodeficiency virus type 1 model studies. *Nucleic Acids Res.* **18**, 999–1005 (1990).

L1. Leong, D. E., Loeffelholz, M. J., Purohite, A., and White, T. J., Detection and identification of pathogenic bacteria using the polymerase chain reaction. *Abstr. Conf. Nucleic Acids, 5th, 1990,* San Diego, Nov. 14–16 (1990).

L2 Lin, L., Gong, Y., Cimino, G. D., Hearst, J. E., and Isaacs, S. T., Two novel, rapid, high yield sample preparation methods for the PCR *Abstr Conf Nucleic Acids, 5th, 1990,* San Diego, Nov. 14–16 (1990).

L3. Longo, M. C., Berninger, M. S., and Harley, J. L., Use of uracil DNA glycosylase to control carryover contamination in polymerase chain rections. *Gene* **93**, 125–128 (1990)

L4. Lopez, M., Ortego, C., Cangalaya, M. A., Inga, R. M., and Arevalo, J. L., Detection of *Leishmania braziliensis* from minimally processed human biopsies via the polymerase chain reaction (PCR) *Abstr Am Soc Microbiol*, May 13–17 (1990)

M1. Mabilat, C., and Courvalin, P., Development of "Oligotyping" for characterization and molecular epidemiology of TEM β-lactamases in *Enterobacteriaceae Antimicrob Agents Chemother* **34**, AAC 230–290 (1990)

M2 Mack, D. H., and Sninsky, J. J., A sensitive method for the identification of uncharacterized viruses related to known virus groups: *Hepadnavirus* model system *Proc. Natl Acad Sci U.S.A* **85**, 6977–6981 (1988)

M3. Malloy, D. C., Nauman, R. K., and Paxton, H., Detection of *Borrelia burgdorferi* using the polymerase chain reaction *J Clin Microbiol* **28**, 1089–1093 (1990)

M4. McLaughlin, G. L., Vodkin, M. H., and Huizinga, H. W., Amplification of repetitive DNA for the specific detection of *Naegleria fowleri* *J. Clin. Microbiol.* **29**, 227–230 (1991).

M5 Mitchell, T. G., White, T. J., and Taylor, J. W., Comparison of ribosomal DNA sequences among the basidiomycetous yeast genera *Cystofilobasdium, Filobasdium,* and *Filobasidiella Mycologia* (in press).

M6. Moser, D. R., Cook, G. A., Ochs, D. E., Bailey, C. P., McKane, M. R., and Donelson, J. E., Detection of *Trypanosoma congolense* and *Trypanosoma brucei* subspecies by DNA amplification using the polymerase chain reaction. *Parasitology* **99**, 57–66 (1989)

M7. Moser, D. R., Kirchhoff, L. V., and Donelson, J. E., Detection of *Trypanosoma cruzi* by DNA amplification using the polymerase chain reaction *J. Clin Microbiol* **27**, 1477–1482 (1989)

M8. Mullis, K. B., Faloona, F. A., Scharf, S., Saiki, R., Horn, G., and Erlich, H., Specific enzymatic amplification of DNA *in vitro*: The polymerase chain reaction *Cold Spring Harbor Symp Quant Biol* **51**, 263–273 (1986)

M9 Mullis, K. B., and Faloona, F. A., Specific synthesis of DNA *in vitro* via a polymerase catalysed chain reaction *In* "Methods in Enzymology" (R. Wu, ed.). Vol. 155, pp. 335–350 Academic Press, San Diego, California, 1987.

M10 Murakami, K., Minamide, W., Wada, K., Nakamura, E., Teraoka, H., and Watanabe, S., Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction *J Clin Microbiol* **29**, 2240–2244 (1991)

M11. Musser, J. M., Schlievert, P. M., Chow, A. W., Ewan, P., Kreiswirth, B. N., Rosdahl, V. T., Naidu, A. S., Witte, W., and Selander, R. K., A single clone of *Staphylococcus aureus* causes the majority of cases of toxic shock syndrome *Proc. Natl. Acad Sci U.S.A* **87**, 225–229 (1990)

M12. Myers, T. W., and Gelfand, D. H., Reverse transcription and DNA amplification by a *Thermus thermophilus* DNA polymerase. *Biochemistry* **30**, 7661–7666.

N1. Newton, C. R., Graham, A., Heptinstall, L. E., Powell, S. J., Summers, C., Kalsheker, N., Smith, J. C., and Markham, A. F., Analysis of any point mutation in DNA. The amplification of refractory mutation systems (ARMS). *Nucleic Acids Res* **17**, 2503–2516 (1989)

N2 Nickerson, D. A., Kaiser, R., Lappin, S., Stewart, J., Hood, L., and Landegren, U., Automated DNA diagnostics using an ELISA-based oligonucleotide ligation assay. *Proc. Natl Acad Sci U.S.A* **87**, 8923–8929 (1990)

N3. Nunberg, J. H., Wright, D. K., Cole, G. E., Petrovskis, E. A., Post, L. E., Compton, T., and Gilbert, J., Identification of the thymidine kinase gene of feline herpes virus Use of degenerate oligonucleotides in the polymerase chain rection to isolate herpes virus gene homologs *J Virol.* **63**, 3240–3249 (1989)

O1. Olive, D. M., Detection of enterotoxigenic *Escherichia coli* after polymerase chain reaction amplification with a thermostable DNA polymerase. *J. Clin. Microbiol.* 27, 261–265 (1989).
O2. Ou, C.-Y., McDonough, S. H., Cabanas, D., Ryder, T. B., Harper, M., Moore, J., and Schochetman, G., Rapid and quantitative detection of enzymatically amplified HIV-1 DNA using chemiluminescent oligonucleotide probes. *AIDS Res. Hum. Retroviruses* 6, 1323–1329 (1990).
O3. Ounissi, H., Derlot, E., Carlier, C., and Courvalin, P., Gene homogeneity for aminoglycoside-modifying enzymes in gram-positive cocci. *Antimicrob. Agents Chemother.* 34, 2164–2168 (1990).
P1. Pao, C. C., Yen, T. S. B., You, J.-B., Maa, J.-S., Fiss, E. H., and Chang, C.-H., Detection and identification of *Mycobacterium tuberculosis* by DNA amplification. *J. Clin. Microbiol.* 28,1877–1880 (1990).
P2. Persing, D. H., and Landry, M. L., *In vitro* amplification techniques for the detection of nucleic acids: New tools for the diagnostic laboratory. *Yale J. Biol. Med.* 62, 159–171 (1989).
P3. Persing, D. H., Telford, S. R., III, Spielman, A., and Barthold, S. W., Detection of *Borrelia burgdorferi* infection in *Ixodes dammini* ticks using PCR. *J. Clin. Microbiol.* 28, 566–572 (1990).
P4. Persing, D. H., Telford, S. R., III, Rys, P. N., Dodge, D. E., White, T. J., Malawista, S. E., and Spielman, A., Detection of *Borrelia burgdorferi* DNA in museum specimens of *Ixodes dammini* Ticks. *Science* 249, 1420–1423 (1990).
P5. Pollard, D. R., Tyler, S. D., Ng, C. W., and Rozee, K. R., A polymerase chain reaction (PCR) protocol for the specific detection of *Chlamydia* spp. *Mol. Cell. Probes* 3, 383–389 (1989).
R1. Reiss, J., and Cooper, D. N., Application of the polymerase chain reaction to the diagnosis of human genetic disease. *Hum. Genet.* 85, 1–8 (1990).
R2. Relman, D. A., Loutit, J. S., Schmidt, T. M., Falkow, S., and Tompkins, L. S., The agent of bacillary angiomatosis: An approach to the identification of uncultivable pathogens. *N. Engl. J. Med.* 323, 1573–1580 (1990).
R3. Rogall, T., Wolters, J., Flohr, T., and Bottger, E., Toward a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*. *Int. J. Syst. Bacteriol.* 40, 323–330 (1990).
R4. Rosa, P. A., and Schwan, T. G., A specific and sensitive assay for the lyme disease spirochete *Borrelia burgdorferi* using the polymerase chain reaction. *J. Infect. Dis.* 160, 1018–1029 (1989).
R5. Rosa, P. A., Hogan, D., and Schwan, T. G., PCR analyses identify two distinct classes of the Lyme disease spirochete *Borrelia burgdorferi*. *J. Clin. Microbiol.* 29, 524–532 (1991).
R6. Rotbart, H. A., Diagnosis of enteroviral meningitis with the polymerase chain reaction. *J. Pediatr.* 117, 85–89 (1990).
S1. Saiki, R. K., Scharf, S. J., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N., Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230, 1350–1354 (1985).
S2. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A., Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239, 487–491 (1988).
S3. Saiki, R. K., Walsh, D. S., and Erlich, H. A., Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. *Proc. Natl. Acad. Sci. U.S.A.* 86, 6230–6234 (1989).
S4. Sakanari, J. A., Staunton, C. E., Eakin, A. E., Craik, C. S., and McKerrow, J. H., Serine proteases from nematode and protozoan parasites: Isolation of sequence homologs using generic molecular probes. *Proc. Natl. Acad. Sci. U.S.A.* 86, 4863–4867 (1989).
S5. Sanchez-Pescador, R., Stempien, M. S., and Urdea, M. S., Rapid chemiluminescent nucleic acid assays for detection of TEM-1 beta-lactamase-mediated penicillin resistance in *Neisseria gonorrhoeae* and other bacteria. *J. Clin. Microbiol.* 26, 1934–1938 (1988).
S6. Sarkar, G., and Sommer, S., Shedding light on PCR contamination. *Nature (London)* 343, 27 (1990).
S7. Sarkar, G., and Sommer, S., More light on PCR contamination. *Nature (London)* 347, 340–341 (1990).
S8. Scanlon, K. J., and Kashani-Sabet, M., Utility of the polymerase chain reaction in detection of gene expression in drug-resistant human tumors. *J. Clin. Lab. Anal.* 3, 323–329 (1989).
S9. Sheppard, H. W., Ascher, M. S., Busch, M. P., Sohmer, P. S., Stanley, M., Lutz, M. C., Chimera, J. A., Madej, R., Rodgers, G. C., Lynch, C., Khayam-Bashi, H., Murphy, E. E., Jr., Eble, B., Bradford, W. Z., Royce, R. A., and Winkelstein, W., Jr., A multicenter proficiency trial of gene amplification (PCR) for the detection of HIV-1. *J. AIDS* 4, 277–284 (1991).
S10. Sjöbring, J., Mecklenburg, M., Andersen, A. B., and Mörner, H., Polymerase chain reaction for detection of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 28, 2200–2204 (1990).
S11. Starnbach, M. N., Falkow, S., and Tompkins, L. S., Species-specific detection of *Legionella pneumophila* in water by DNA amplification and hybridization. *J. Clin. Microbiol.* 27, 1257–1261 (1989).
T1. Tannich, E., and Burchard, G. D., Differentiation of pathogenic from nonpathogenic *Entamoeba histolytica* by restriction fragment analysis of a single gene amplified *in vitro*. *J. Clin. Microbiol.* 29, 250–255 (1991).
T2. Tzianabos, T., Anderson, B. E., and McDade, J. E., Detection of *Rickettsia rickettsii* DNA in clinical specimens by using polymerase chain reaction technology. *J. Clin. Microbiol.* 27, 2866–2868 (1989).
V1. Victor, T., DuToit, R., Van Zyl, J., Bester, A. J., and Van Helden, P. D., Improved method for the routine identification of toxigenic *Escherichia coli* by DNA amplification of a conserved region of the heat-labile toxin A subunit. *J. Clin. Microbiol.* 29, 158–161 (1991).
V2. Vilgalys, R., and Hester, M., Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J. Bacteriol.* 172, 4238–4246 (1990).
W1. Wakefield, A. E., Pixley, F. J., Banerji, S., Sinclair, K., Miller, R. F., Moxon, E. R., and Hopkin, J. M., Amplification of mitochondrial ribosomal RNA sequences from *Pneumocystis carinii* DNA of rat and human origin. *Mol. Biochem. Parasitol.* 43, 69–76 (1990).
W2. Wakefield, A. E., Pixley, F. J., Banerji, S., Sinclair, K., Miller, R. F., Moxon, E. R., and Hopkin, J. M., Detection of *Pneumocystis carinii* DNA amplification. *Lancet* 336, 451–453 (1990).
W3. Wang, A. M., Doyle, M. V., and Mark, D. F., Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci. U.S.A.* 86, 9717–9721 (1989).
W4. Waters, A. P., and McCutchan, T. F., Rapid, sensitive diagnosis of malaria based on ribosomal RNA. *Lancet* June 17, pp. 1343–1346 (1989).
W5. Webb, L., Carl, M., Malloy, D. C., Dasch, G. A., and Azad, A. F., Detection of murine typhus infection in fleas by using the polymerase chain reaction. *J. Clin. Microbiol.* 28, 530–534 (1990).
W6. Welch, D., Lee, C.-H., and Larsen, S. H., Detection of plasmid DNA from all *Chlamydia trachomatis* serovars with a two-step polymerase chain reaction. *Appl. Environ. Microbiol.* 56,2494–2498 (1990).
W7. White, T. J., Arnheim, N., and Erlich, H. A., The polymerase chain reaction. *Trends Genet.* 5, 185–189 (1989).
W8. White, T. J., Bruns, T. D., Lee, S., and Taylor, J. W., Analysis of phylogenetic relationships by amplification and direct sequencing of ribosomal RNA. *In* "PCR Protocols: A Guide to Methods and Applications" (M. A. Innis, D. H. Gelfand, D. H. Sninsky, J. J. Sninsky, and T. J. White, eds.), pp. 315–322. Academic Press, San Diego, California, 1990.

W9. Williams, S. D., and Kwok, S., Polymerase chain reaction: Applications for viral detection. *In* "Laboratory Diagnosis of Infections" (E. H. Lennette, ed.), pp. 147–173. Dekker, New York, 1991.
W10. Wilson, K. H., Blitchington, R., Shah, P., McDonald, G., Gilmore, R. D., and Mallavia, L. P., Probe directed at a segment of *Rickettsia rickettsii* rRNA amplified with polymerase chain reaction. *J. Clin. Microbiol.* 27, 2692–2696 (1989).
W11. Witte, J. J., Update: Transmission of HIV infection during an invasive dental procedure—Florida. *Morbid. Mortal. Week. Rep* 40, 21–27 (1990).
W12. Wittwer, C. T., Fillmore, G. C., and Garling, D. J., Minimizing the time required for DNA amplification by efficient heat transfer to small samples. *Anal. Biochem* 186, 328–331 (1990).
W13. Wordsworth, B. P., Hughes, R. A., Keat, A. C., and Bell, J. I., Chlamydial DNA is absent from the joints of patients with sexually acquired reactive arthritis. *Br. J. Rheumatol.* 29, 208–210 (1990).
W14. Wren, B., Clayton, C., and Tabaqchali, S., Rapid identification of toxigenic *Clostridium difficile* by polymerase chain reaction. *Lancet* 335, 423 (1990).
W15. Wu, D. Y., Ugozzoli, L., Pal, B. K., and Wallace, R. B., Allele-specific enzymatic amplification of β-globin genomic DNA for diagnosis of sickle cell anemia. *Proc. Natl. Acad. Sci. U.S.A.* 86, 2757–2760 (1989).
Y1. Yamada, M., Hudson, S., Tournay, O., Bittenbender, S., Shane, S. S., Lange, B., Yoshihide, T., Caton, A., and Rovera, G., Detection of minimal disease in hematopoietic malignancies of the B-cell lineage by using third-complementarity-determining region (CDR-III)-specific probes. *Proc. Natl. Acad. Sci. U.S.A.* 86, 5123–5127 (1989).
Y2. Yap, E. P. H., and O'D.McGee, J., Short PCR product yields improved by lower denaturation temperatures. *Nucleic Acids Res.* 19, 1713 (1991).
Z1. Zolg, J. W., Chen, G.-X., and Plitt, J. R., Detection of pyrimethamine resistance in *Plasmodium falciparum* by mutation-specific polymerase chain reaction. *Mol. Biochem. Parasitol* 39, 257–266 (1990).

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ONCOGENES IN CANCER

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